

INDIAN AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

I. A. R. I. 6. MGIPC—S4—10 AR—21.6.49—1,006.

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A, HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITORIAL BOARD

RUDOLPH J. ANDERSON
REGINALD M. ARCHIBALD
W. MANSFIELD CLARK
HANS T. CLARKE
CARL F. CORI
EDWARD A. DOISY
JOSEPH S. FRUTON
WENDELL H. GRIFFITH

A. BAIRD HASTINGS
CHIBALD HOWARD B. LEWIS
CLARK ELMER V. McCOLLUM
ESMOND E. SNELL
WARREN M. SPERRY
EDWARD L. TATUM
HUBERT B. VICKERY
FITH VINCENT DU VIGNEAUD
HARLAND G. WOOD

VOLUME 183
BALTIMORE
1950

COPYRIGHT, 1950

BI

THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

PUBLISHED AT YALE UNIVERSITY FOR
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, INC.
BALTIMORE 2, U.S.A.

CONTENTS OF VOLUME 183

No. 1, March, 1950

HITCHINGS, GEORGE H., ELION, GERTRUDE B., FALCO, ELVIRA A., RUSSELL,	LUL
PETER B., SHERWOOD, MARION B., and VANDERWERFF, HENRY. An-	
tagonists of nucleic acid derivatives. I. The Lactobacillus casei model	1
CAGAN, RALPH N., GRAY, JOHN L., and JENSEN, H. The influence of certain	•
endocrine secretions on amino acid oxidase	11
GEREN, WILLIAM, BENDICH, AARON, BODANSKY, OSCAR, and BROWN, GEORGE	
Bosworth. The fate of uric acid in man	21
LITTLE, HENRY N., and BLOCH, KONRAD. Studies on the utilization of acetic	
acid for the biological synthesis of cholesterol.	33
ENGEL, LEWIS L., PATTERSON, HELEN R., WILSON, HILDEGARD, and SCHINKEL,	-
MARION. The colorimetric estimation of urinary neutral steroid alcohols	47
VESTLING, CARL S., and KNOEPFELMACHER, AUGUSTA A. Lactic dehydrogenase	
of liver and its relation to thyroid activity in the rat	63
COPENHAVER, J. H., JR., McShan, W. H., and MEYER, ROLAND K. The de-	
termination of glutamic acid dehydrogenase in tissue homogenates	73
PATTERSON, JOHN W. The diabetogenic effect of dehydroascorbic and dehydro-	
isoascorbic acids	81
EICHEL, BERTRAM, WAINIO, W. W., PERSON, P., and COOPERSTEIN, S. J. A	
partial separation and characterization of cytochrome oxidase and cyto-	
chrome b	89
HAMMARSTEN, EINAR, REICHARD, PETER, and SALUSTE, E. Pyrimidine nucleo-	
sides as precursors of pyrimidines in polynucleotides	105
RABINOVITZ, M., and BOYER, PAUL D. The inhibition of the succinoxidase	
system by α -tocopheryl phosphate and sodium dodecyl sulfate	111
SCHNEIDER, WALTER C., and HOGEBOOM, GEORGE H. Intracellular distribution	
of enzymes. V. Further studies on the distribution of cytochrome c in rat	
live homogenates	123
HEPPEL, LEON A., and HILMOE, R. J. Metabolism of inorganic nitrite and	
nitrate esters. II. The enzymatic reduction of nitroglycerin and erythritol	
tetranitrate by glutathione	129
STEVENS CARL M., and Bush, Jess A. New syntheses of α-amino-ε-guanidino-	
n-caproic acid (homoarginine) and its possible conversion in vivo into lysine.	139
Kunkel, H. O., and Nelson, Walter L. The effect of bixin and carotene on the	
oxidation of methyl linoleate	
SCHUBERT, JACK, and WALLACE, HERSCHEL, JR. The effect of zirconium and	
sodium citrate on the distribution and excretion of simultaneously injected	
thorium and radiostrontium	
MARLOW, H. W. Groups involved in the Zimmermann and Kober reactions	167
ROTH, JAT S., and Allison, James B. The effects of feeding excess di-methio-	
nine and choline chloride to rats on a casein diet.	
McGilvery, R. W., and Cohen, Philip P. Enzymatic synthesis of ornithuric	
acids	
SIMMONDS, SOFIA, KELLER, ELIZABETH B., CHANDLER, JOSEPH P., and DU	
VIGSEAUD, VINCENT. The effect of ethionine on transmethylation from	
methionine to choline and creatine in vivo	191

IV CONTENTS

HARGER, R. N., RANEY, B. B., BRIDWELL, E. G., and KITCHEL, MARY F. The partition ratio of alcohol between air and water, urine and blood; estimation	ı
and identification of alcohol in these liquids from analysis of air equilibrated with them	
Schulman, John, Jr., and Keating, Richard P. Studies on the metabolism of thiourea. I. Distribution and excretion in the rat of thiourea labeled with radioactive sulfur.	
STÄLLBERG-STENHAGEN, STINA, and STENHAGEN, EINAR. Studies on hydrocarbons structurally related to phthiocerol. Synthesis of the levorotatory	
enantiomorph of 4-methyltritriacontane	22 3
Helmreich, M. L. Metabolism of testosterone by livers of different species of animals	231
bovine and equine immune proteins Furst, Sidney S., Roll, Paul M., and Brown, George Bosworth. On the	241
renewal of the purines of the desoxypentose and pentose nucleic acids Bendich, Aaron, Brown, George Bosworth, Philips, Frederick S., and	251
THIERSCH, J. B. The direct oxidation of adenine in vivo VAN PILSUM, JOHN F., and BERG, CLARENCE P. The comparative availabilities	267
of mixtures of the L and DL modifications of the essential amino acids for growth in the rat.	279
CANZANELLI, ATTILIO, RAPPORT, DAVID, and GUILD, RUTH. The effect of previous carbohydrate deprivation on the synthesis of alanine by liver in vitro KLENDSHOJ, NIELS C., FELDSTEIN, MILTON, and SPRAGUE, ALICE L. The spec-	291
trophotometric determination of carbon monoxide	297
VINCENT P., EMERSON, KENDALL, JR., and ARCHIBALD, REGINALD M. Measurement of specific gravities of whole blood and plasma by standard copper sulfate solutions	305
VAN SLYKE, DONALD D., HILLER, ALMA, PHILLIPS, ROBERT A., HAMILTON, PAUL B., DOLE, VINCENT P., ARCHIBALD, REGINALD M., and EDER, HOWARD A. The estimation of plasma protein concentration from plasma specific	
gravityVan Slyke, Donald D., Phillips, Robert A., Dole, Vincent P., Hamilton,	331
PAUL B., ARCHIBALD, REGINALD M., and PLAZIN, JOHN. Calculation of hemoglobin from blood specific gravities	349
stituted indoleacetic acids as plant hormones in the pea test	361
I. Isolation of 17-hydroxy-11-dehydrocorticosterone and other compounds from the urine of normal males	365
Transphosphorylation by alkaline phosphatase in the absence of nucleotides. KIELLEY, W. WAYNE, and MEYERHOF, OTTO. Studies on adenosinetriphosphatase of muscle. III. The lipoprotein nature of the magnesium-activated	377
adenosinetriphosphatase	391
No. 2, April, 1950	
ISELIN, BEAT M., HUANG, H. T., and NIEMANN, CARL. A colorimetric method for the determination of chymotrypsin activity	403

CONTENTS

SINGER, THOMAS P., and KEARNEY, EDNA B. The non-enzymatic reduction of cytochrome c by pyridine nucleotides and its catalysis by various flavins	409
Pihl, Alexander, and Bloch, Konrad. The relative rates of metabolism of neutral fat and phospholipides in various tissues of the rat	431
Pihl, Alexander, Bloch, Konrad, and Anker, H. S. The rates of synthesis of fatty acids and cholesterol in the adult rat studied with the aid of labeled acetic acid	
Reed, Lester J. The occurrence of γ-aminobutyric acid in yeast extract; its isolation and identification	
McConnell, Kenneth P., and Cooper, Betty J. Distribution of selenium in serum proteins and red blood cells after subcutaneous injection of sodium selenate containing radioselenium.	
SHANTZ, EDGAR M., and BRINKMAN, JOHN H. Biological activity of pure vitamin A2.	
KERESZTESY, JOHN C., and SILVERMAN, MILTON. Partial purification of a factor essential for growth of Leuconostoc citrovorum	4 73
HOFMANN, KLAUS, DICKEL, DANIEL F., and AXELROD, A. E. The preliminary characterization of two biotin-containing fractions in beef liver	481
DISCHE, ZACHARIAS. A modification of the carbazole reaction of hexuronic acids for the study of polyuronides	489
with analogues of pimelic acid	495
rabbit erthrocytes: the existence of a diphosphoglycerate mutase	507
rat, studied with isotopic lactate. LORBER, VICTOR, LIFSON, NATHAN, SAKAMI, WARWICK, and WOOD, HARLAND G. Conversion of propionate to liver glycogen in the intact rat, studied with	517
isotopic propionate	531
upon enzyme activity in the rat	
phosphoric ester in rat organs and human tumors	
deficient in pyruvic oxidase	549 561
WOODRUFF, H. BOYD, and FOSTER, JOHN C. Analysis for vitamin B ₁₂ and vitamin B _{12a} by paper strip chromatography	
GREENMAN, LAWRENCE. Alterations in blood glucose following intravenous galactose	
POTTER, RICHARD L., and ELVEHJEM, C. A. On the activity of oxybiotin	587
JORPES, J. ERIK, BOSTRÖM, HARRY, and MUTT, VIKTOR. The linkage of the	593
amino group in heparin	607
the methyl group of dietary methionine.	617

VI CONTENTS

Keller, Elimbeth B, Boissonnas, R A, and Du Vigneaud, Vincent The	
origin of the methyl group of epinephrine	627
BACHER, JAMES L and ALLEN, FRANK WORTHINGTON The composition of	
certain fractions obtained by the action of ribonuclease on ribonucleic acid	
from yeast	633
Bacher, Jamis Σ , and Allen, Frank Worthington A comparison of pentose	
nucleic acid from pancreas with ribonucleic acid from yeast	641
Peterson, D W Some properties of a factor in alfalfa meal causing depression	
of growth in chicks	647
MACKENZIE, JULIA B ROSENKRANTZ, HARRIS, ULICK, STANLEY, and MILHO	
RAT, ADE T The biological activity of a tocopherylhydroquinone and	
α tocopherylquinone	655
ROSENFELD MORRIS and SURGENOR, DOUGLAS M Methemalbumin Inter-	
action between human serum albumin and ferriprotoporphyrin IX	663
GLAZKO, ANTHONY J DILL, WESLEY A, and REBSTOCK, MILDRED C BIO	
chemical studies on chloramphenicol (Chloromycetin) III Isolation and	
identification of metabolic products in urine	679
NAGANNA L Liythrocyte pyrophosphatase in health and disease II Re	
versible and irreversible inactivations of the enzyme	693
SPILS, JOSEPH R, and CHAMBERS, DORRIS C Utilization of optical isomers of	
methionine and formylmethionine by some lactobacilli	709
Granick, S Magnesium vinyl pheoporphyrin a_5 another intermediate in the	
biological synthesis of chlorophyll	713
LUBSCHEZ, ROSF Chemical determination of histamine in human blood	731
RODNLY, GERTRUDE, SWANSON, ANN L, WHEELER, L M, SMITH, GRANT N,	
and Worrei, Ciclia S The effect of a series of flavonoids on hyaluroni-	
dase and some other related enzymes	739
LONDON, IRVING M SHEMIN, DAVID, and RITTENBERG, D Synthesis of heme	= 40
in vitro by the immature non nucleated mammalian erythrocyte	749
SHEMIN, DAVID, LONDON, IRVING M, and RITTENBERG, D The synthesis of	
protoporphyrin in vitro by red blood cells of the duck	757
SHEMIN, DAVID, LONDON, IRVING M, and RITTENBERG, D The relationship of	=0=
serine to porphyrin synthesis	767
BADDILEY, J, EHRENSVARD, G, JOHANSSON, R, REIO, L, SALUSTE, E, and	
STJERNHOIM, R Acetic acid metabolism in Torulopsis utilis I The	~~1
cultivation of Torulopsis yeast on C ¹³ H ₂ C ¹⁴ OOH as the single carbon source	771
BADDILEY, J, EHRENSVARD, G, KLEIN, E, REIO, L, and SALUSTE, E Acetic	
acid metabolism in Torulopsis utilis II Metabolic connection between	
acetic acid and tyrosine and a method of degradation of the phenolic ring structure in tyrosine	777
~ _ · · · .	777
STEINHARDT, JACINTO, and ZAISER, ETHEL M Combination of wool protein with cations and hydroxyl ions	79A
	789 803
Index to Volume 183	5U3

CORRECTION

On page 90, line 11, Vol. 181, No. 1, November, 1949, read 113-115° for 213-215°.

On page 764, legend to Fig. 3, Vol. 181, No. 2, December, 1949, read 3.375 for 3375 and 2.85 for 285.

Linlithgow Library.

Imperial Agricultural Research Institute

New Delhi.

ANTAGONISTS OF NUCLEIC ACID DERIVATIVES

I. THE LACTOBACILLUS CASEI MODEL

By GEORGE H. HITCHINGS, GERTRUDE B. ELION, ELVIRA A. FALCO, PETER B. RUSSELL, MARION B. SHERWOOD, AND HENRY VANDERWERFF

(From the Wellcome Research Laboratories, Tuckahoe, New York)

(Received for publication, June 11, 1949)

A comprehensive study of the relationship between chemical structure and the rôle of pyrimidines and related substances in the biosynthesis of derivatives of nucleic acids was undertaken a number of years ago. Several preliminary accounts of this work have been given (1-5).

It was expected that such studies might serve two purposes. The biochemical pathways involved in nucleic acid synthesis might be revealed if substances could be found to block these reactions. Reversal studies might then result in the identification of previously unknown biochemical intermediates. This approach has come to be regarded with some skepticism (6) since reversals by obviously unnatural substances are not infrequently observed. Nevertheless it is felt that interpretations based on the above premises are worth consideration since frequently biochemical mechanisms are suggested which can be studied by more definitive methods.

The second purpose of these studies was the discovery of new chemotherapeutic agents. The rate of nucleic acid synthesis by parasitic tissues of all kinds, whether of bacteria, rickettsiae, viruses, protozoa, or neoplasms, is apparently more rapid than that of host tissues as a consequence of the rapid rate of reproduction upon which the parasite depends for survival. An antagonist might, therefore, be expected to affect the parasite more strongly than the host on a purely differential rate basis. This type of effect probably explains the protective action of diaminopurine (7) and the structural analogues of folic acid (8) in leucemia.

A more important consideration for the elaboration of chemotherapeutic agents on this basis lies in the qualitative differences in the pathways to nucleic acid synthesis which may exist. When this work was begun, a considerable number of microorganisms were known to require preformed pyrimidines and purines for growth (9), whereas uracil, thymine, and guanine had been found to play no rôle in nucleic acid synthesis of the rat (10). Subsequently, it has been discovered that adenine (11), diaminopurine (12), and orotic acid (13) contribute to the nitrogenous fractions

¹Elion, G. B., Falco, E. A., and Hitchings, G. H., to be published.

of nucleic acid in the rat. However, qualitative differences in the utilization of various pyrimidines and purines appear to exist between the mammal and a considerable variety of bacteria. The related requirements of protozoa, rickettsiae, and viruses are relatively unknown. Tetrahymena geleii utilizes guanine and uracil (14), requirements similar to those of molds (15) and bacteria (9) and unlike those of the rat. Plasmodium gallinaceum appears to resemble Lactobacillus casei in some of its requirements since a new type of antimalarial has been found among the antagonists studied in the L. casei screening test (16). Relatively little is known about the nitrogenous bases involved in virus multiplication, although adenine may be involved in the reproduction of the Escherichia coli T₂ phage (17). The multiplication of vaccinia virus in vitro is inhibited by a number of antimetabolites (18). Some difference between this virus and mammalian tissues in the biochemical pathways leading to nucleic acid may be indicated by the fact that virus multiplication is inhibited by diaminopurine but, in contrast to many other tissues, is relatively unaffected by the structural analogues of folic acid (18).

L. casei was chosen as a test organism for the preliminary screening of substances for activity as antagonists of nucleic acid synthesis. This choice was based on the observations of Snell and Mitchell (19) and Stokstad (20) that the folic acid requirement of this microorganism is closely related to thymine and purine synthesis, since the vitamin requirement could be replaced by these substances. This relationship allows the study of a potential antimetabolite in a variety of ways in the same microorganism.

EXPERIMENTAL

Medium—The basal medium (O) is that of Landy and Dicken (21) with omission of purines, uracil, and folic acid and supplemented or modified as follows (amounts per 100 ml. of medium): DL-alanine 20 mg., DL-glutamic acid 0.5 mg., pyridoxine hydrochloride 200 γ, p-aminobenzoic acid 20 mg., thiamine chloride 20 γ, glucose 1.5 gm., sodium acetate 1.0 gm., MgSO₄·7H₂O 20 mg., FeSO₄·7H₂O 4 mg., and MnSO₄·2H₂O 4 mg.

100 γ of thymine (T) or 4.6 mµgm. of folic acid (FA) per 100 ml. may be added to produce three test media (O, OT, OFA). The addition of purine (P) to the basal medium, usually 1 mg. of adenine sulfate per 100 ml., adds three modifications (P, PT, PFA), giving six test media in all (cf. Table I).

Growth in these media is relatively slow, but this avoids several complications which appear when growth-accelerating factors are added. For example, growth is faster when enzymatic rather than acid casein hy-

drolysate is used. Under these conditions uracil becomes a limiting factor for growth, but can be omitted altogether from the regular medium. This observation has been used as the basis for a study of substances with anti-uracil effects (to be published).

Inoculum—Seed cultures are grown as recommended by Snell and Strong (22), twice washed with saline, centrifuged, resuspended in saline, and

Table I
Response of L. casei to Pyrimidine Derivatives

Line No.	Substance	Substance Concentration					Acid production in various media, ml. of 0.1 N per 10 ml.					
			0	от	OFA	P	PT	PFA				
		mg. per 10 ml.										
1	Control		0.6	0.8	6.0	1.0	6.5	8.0				
2	Adenine sulfate	0.1	1.0	6.5	8.0	1.0	6.8	8.8				
3	2,4-Diamino-5-formamido- 6-hydroxypyrimidine	1.0	0.8	3.9	7.0	1.0	6.5	8.1				
4	Thymine	0.01	0.8	0.8	5.8	6.5	6.6	10.0				
5	5-Methyl-4-hydroxypyrimi- dine	1.0	0.9	0.9	5.5	7.0	6.5	10.2				
6	Folic acid	0.46×10^{-6}	6.0	5.8	8.0	8.0	10.0	10.5				
7	5-Hydroxyuracil	1.0	0.5	0.6	0.5	0.6	0.5	0.6				
8	5-Aminouracil	1.0	0.3	0.5	1.0	0.4	3.3	3.2				
9	5-Nitrouracil	1.0	0.3	0.6	0.5	0.5	6.0	0.8				
10	5-Bromouracil	1.0	0.5	0.5	4.8	2.0	2.5	10.0				
11	4-Aminofolic acid	0.5×10^{-6}	0.5	0.5	0.4	0.5	5.6	2.0				
12	2,4-Diamino-5-p-chloro- phenoxypyrimidine	0.01	0.4	0.4	0.3	0.5	4.0	0.8				
13	2,4-Diamino-5,6-dimethyl- pyrimidine	1.0	0.2	0.2	0.2	0.3	4.2	0.5				
14	2,6-Diaminopurine	1.0	0.3	0.3	0.8	1.1	6.5	7.0				

O, basal medium; OT, O + 1 γ of thymine per ml.; OFA, O + 0.046 mµgm. of folic acid per ml.; P, O + 10 γ of adenine sulfate per ml.; PT, P + 1 γ of thymine per ml.; and PFA, P + 0.046 mµgm. of folic acid per ml.

0.02 ml. added to 10 ml. of medium. The carry-over of folic acid appears to be much less than in an inoculum prepared from a culture grown in the medium (O) supplemented by 1.0 mµgm. of folic acid per ml., since use of the latter inoculum gave smaller effects of folic acid antagonists (to be published).

Growth—The growth is expressed as ml. of 0.1 N acid produced during 68 hours of incubation at 37°. An exhaustive comparison of this procedure

with other possible criteria of growth such as plate counts, turbidities, cell counts, and nitrogen determinations has been made.² For the purposes of this paper it is sufficient to state that lactic acid production is at least as satisfactory as any other single criterion of growth.

Results

The growth characteristics of *L. casei* under the conditions described are shown in Figs. 1 and 2. Fig. 1 shows the lactic acid production as related to the concentration of folic acid at three time intervals. It will be noted that lactic acid production is related to the folic acid concentration over the range of 0.023 to 0.575 mµgm. per ml. but that increases in folic

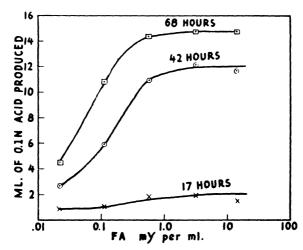


Fig. 1. Lactic acid production as affected by concentration of folic acid (FA)

acid concentration above this level have essentially no further effect. At 68 hours and with 0.575 m μ gm. of folic acid per ml., the lactic acid found approaches the stoichiometric amount obtainable from the glucose present. At all levels of folic acid, a considerable lag period in fermentation is shown.

For routine use, the concentration of folic acid was chosen so that the growth curve in this medium (OFA) approximates that in the medium containing thymine and adenine (PT). This is shown in Fig. 2 where the growth curves in these two media are also contrasted with that of a medium containing sufficient folic acid to produce a maximal response. Addition of further thymine does not increase the rate of lactic acid production above that observed with 10γ per 10 ml. (23). However, the conversion of glucose to lactic acid in this medium is not limited to half maximal (23)

²Lorz D, Hitchings, G. H, and VanderWerff, H, to be published.

but is simply slower than when optimal folic acid is present. This slower rate of fermentation is not a function of the number of organisms, the number of viable organisms, or the total bacterial nitrogen,² but seems to reflect a metabolic difference.

The use of six minor variations of the same basal medium to detect as many types of biological activity is illustrated in Table I. The basal medium (O) is supplemented with adenine (P), thymine (OT), or folic acid (OFA) and combinations of adenine and thymine (PT) and adenine and folic acid (PFA). When a substance is tested in each of these media it is often possible to determine at once the type of biological activity which

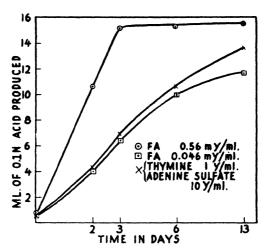


Fig. 2. Lactic acid production of L. casei in various media as a function of time

it possesses. Thus the addition of adenine results in a marked increase in growth in the OT medium and an appreciable stimulation in OFA, while 2,4-diamino-5-formamido-6-hydroxypyrimidine is identified as a purine-like substance because the same pattern of biological activity is followed. The marked difference in growth in the OT medium (without adenine) and the PT medium (with adenine) illustrates the essential nature of the purine requirement when thymine is used as the nutrilite. The growth in the OFA medium shows that the purine requirement can be satisfied from endogenous reactions when folic acid is present.

The effects of the addition of thymine or of a thymine-like substance (5-methyl-4-hydroxypyrimidine) can be illustrated by Lines 4 and 5 of Table I. The activity of such substances is seen primarily in the P medium, again showing that purine is essential for growth with thymine and

demonstrating that 20 γ per 10 ml. of thymine do not increase the rate of growth above that obtained with 10 γ per 10 ml.

The addition of folic acid (Line 6, Table I) results in the stimulation of growth in all media, but this is especially marked in the two control media (O and P).

These media are useful also for the detection of antagonists. For the most part, separate reversal experiments are necessary to determine the exact nature of the inhibition. Thus 5-hydroxyuracil inhibits in all media and its action is reversed by neither folic acid nor thymine³ but is reversed by uracil (5). Similarly, 5-aminouracil inhibits growth with either thymine or folic acid, but its action is partially reversed by purine (the difference between OFA and PFA) and completely by either folic acid or thymine (5).1 5-Nitrouracil (Line 9, Table I) apparently acts as a folic acid antagonist since growth with this substance is completely blocked in both the absence (OFA) and presence (PFA) of purine, while growth with thymine (PT) is essentially unaffected. 5-Bromouracil, on the other hand, shows some tendency to simulate thymine in its effects (increase in P and PFA, decrease in OFA) but clearly blocks the utilization of thymine (decrease in PT). A structural analogue of folic acid (Line 11) resembles nitrouracil in blocking growth with folic acid but leaving growth with thymine relatively unaffected. A phenoxydiaminopyrimidine (Line 12) behaves similarly but has a somewhat greater effect on thymine growth and is somewhat less affected by the presence of purines than is the analogue of folic acid. Diaminodimethylpyrimidine (Line 13) behaves similarly. Reversal of the inhibitory action of 4-aminofolic acid, the diaminopyrimidines, and nitrouracil by folic acid can be demonstrated (5).

The action of 2,6-diaminopurine (Line 14, Table I) is seen to be directed primarily toward growth with folic acid in the absence of purine. This inhibitory activity is readily reversed by adenine (PFA). At high concentrations of the inhibitor such as that illustrated here, this reversal is specific (4, 5), although a number of purines can satisfy the normal purine requirement of the organism. This inhibitor, therefore, gives the appearance of interference with endogenously produced adenine while having little influence on the utilization of exogenously supplied adenine (5).

DISCUSSION

The finding that 2,6-diaminopurine, labeled with isotopic nitrogen in the 1 and 3 positions, can be incorporated into the guanine fraction of the ribonucleic acid of the rat (12), although somewhat beclouding the mechanical results of the rat (12) although somewhat beclouding the mechanical results of the rat (12).

³The action of this substance was erroneously stated earlier (2) to be reversible by these nutrilites.

Elion, G. B., and Hitchings, G. H., to be published.

nism of the inhibitory effects of diaminopurine, does demonstrate the participation of this substance in nucleic acid metabolism. This kind of evidence is not available for other pyrimidine derivatives, but an a priori case for the participation of some of these in nucleic acid metabolism can be argued on the basis of reversal experiments. The fundamental premise of this work that structural analogues of the purines and pyrimidines may be found to affect nucleic acid metabolism appears to be highly probable.

It was recognized early in this work that a substance might fulfil the criteria of competitive antagonism with a metabolite in a single microbial system without possessing the full complement of properties which are found in structural analogues of the metabolite. Thus 5-nitrouracil is not regarded as an "antifolic" in any broad sense, since its action differs from this pattern in microorganisms closely related to *L. casei* and apparently may show different effects in the same organism under different conditions (24). On the other hand, the structural analogues of folic acid show "antifolic" effects in a wide variety of biological systems (25–27).

The demonstration of the true nature of an antagonist thus depends to a considerable extent on the results of studies in a wide variety of biological systems. If a substance produces the symptoms of deprivation of the metabolite in all systems studied, it may safely be assumed that it is acting by displacement of the metabolite from cell receptors which are similar in many organisms and essential to the utilization of the metabolite. On the other hand, a substance may exhibit a consistent pattern of antagonism in a number of microbiological systems and yet fail to exhibit this antagonism in more complex organisms by reason of the intervention of pharmacological effects or metabolic changes which make it impossible to reach the concentration at which the antimetabolite effect would be expected. This explanation may apply, for example, to the lack of antifolic effects of 2,4-diamino-5,6-dimethylpyrimidine which has quite striking pharmacological properties.⁵

A somewhat intermediate classification of antimetabolites includes compounds which have similar but distinct effects in different organisms. Thus, 2,4-diamino-5-p-chlorophenoxypyrimidine gives a clear cut picture of folic acid antagonism in L. casei. It is an antimalarial approximating quinine in activity against P. gallinaceum (16) and this activity is partially reversed by folic acid in vivo.⁶ In T. geleii it is inhibitory in the presence of excess folic acid and this inhibition is reversible by uracil.⁷ No evidence

⁸Philips, F., personal communication.

Greenberg, Joseph, personal communication.

⁷Kidder, G. W., personal communication.

of antifolic effects with this substance has been obtained in either mouse or chick.

A number of possibilities exist for the mode of action of such a substance: (1) The compound may possess several active centers which allow it to block, more or less independently, different biochemical reactions in different tissues; (2) a single reaction may be blocked in all organisms but this reaction may be of relatively greater importance to one organism than to another; (3) the dissociation of the inhibitor-cell receptor complex may vary widely from organism to organism; or (4) the reaction blocked may be one which occurs only sporadically in nature as an obligate biochemical mechanism. Whatever their mode of action, such antimetabolites provide interesting leads, for the successful elaboration of chemotherapeutic agents depends on the discovery of substances which possess considerable biological and biochemical specificity.

SUMMARY

- 1. A screening test is described, with *Lactobacillus casei* as the test object, for the discovery and characterization of substances capable of blocking the biosynthesis of nucleic acid and related substances.
- 2. The use of this screening test in the elaboration of new chemotherapeutic agents is exemplified and discussed.

BIBLIOGRAPHY

- 1. Hitchings, G. H., Elion, G., Falco, E. A., and VanderWerff, H., Abstracts, American Chemical Society, New York, 3 C (1947).
- 2. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., Science, 102, 251 (1945).
- 3. Hitchings, G. H., Elion, G. B., and VanderWerff, H., Federation Proc., 7, 160 (1948).
- Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., J. Biol. Chem., 174, 765 (1948).
- Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and VanderWerff, H., Ann. New York Acad. Sc., in press (1949).
- Hitchings, G. H., Elion, G. B., and VanderWerff, H., J. Biol. Chem., 174, 1037 (1948).
- Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C., Cancer, 2, 119 (1949).
- 8. Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., Cancer, 2, 113 (1949).
- 9. Loring, H. S., Ann. Rev. Biochem., 13, 309 (1944).
- 10. Plentl, A. A., and Schoenheimer, R., J. Biol. Chem., 153, 203 (1944).
- Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., J. Biol. Chem., 172, 469 (1948).
- 12. Bendich, A., and Brown, G. B., J. Biol. Chem., 176, 1471 (1948).
- Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., von Ubisch, H. and Bergstrom, S., J. Biol. Chem., 179, 169 (1949).

HITCHINGS, ELION, FALCO, RUSSELL, SHERWOOD, AND VANDERWERFF

- 14. Kidder, G. W., and Dewey, V. C., Proc. Nat. Acad. Sc., 34, 566 (1948).
- 15. Robbins, W. J., Proc. Nat. Acad. Sc., 29, 201 (1943).
- Falco, E. A., Hitchings, G. H., Russell, P. B., and VanderWerff, H., Nature, 164, 107 (1949).
- 17. Cohen, S. S., and Fowler, C. B., J. Exp. Med., 87, 275 (1948).
- Thompson, R. L., Wilkin, M., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B., Science, 110, 454 (1949).
- 19. Snell, E. E., and Mitchell, H. K., Proc. Nat. Acad. Sc., 27, 1 (1941).
- 20. Stokstad, E. L. R., J. Biol. Chem., 139, 475 (1941).
- 21. Landy, M., and Dicken, D. M., J. Lab. and Clin. Med., 27, 1086 (1942).
- 22. Snell, E. E., and Strong, F. M., Ind. and Eng. Chem., Anal. Ed., 11, 346 (1939).
- 23. Stokes, J. L., J. Bact., 48, 201 (1944).
- 24. Shive, W., Ann. New York Acad. Sc., in press.
- Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., J. Biol. Chem., 169, 427 (1947).
- 26. Hertz, R., Science, 107, 300 (1948).
- 27. Philips, F. S., and Thiersch, J., J. Pharmacol. and Exp. Therap., in press.

THE INFLUENCE OF CERTAIN ENDOCRINE SECRETIONS ON AMINO ACID OXIDASE*

BY RALPH N. CAGAN, JOHN L. GRAY, AND H. JENSEN

(From the Medical Department Field Research Laboratory, Fort Knox, Kentucky)

(Received for publication, July 14, 1949)

Deamination is one step in the metabolism of amino acids. This process is catalyzed by the D- and L-amino acid oxidases. It is known that various endocrine secretions will affect the rate of enzymatic reactions.

The present communication presents data on the interrelation of amino acid concentration in the blood and the secretions of the hypophysis, adrenal, and thyroid on the rate of activity of the liver and kidney amino acid oxidase systems. Alterations in the oxygen uptake, as measured by the Warburg manometric technique, were used as criteria for changes in the enzymatic activity of the amino acid oxidase, AAO.

EXPERIMENTAL

White male rats of the Sprague-Dawley strain, weighing 250 to 300 gm. and fed on Purina dog chow checkers, were employed. They were fasted for 18 hours before the experiment, but were permitted water. Four groups of animals were employed: normal, adrenalectomized, hypophysectomized, and thyroidectomized. Within each group a series of experiments was carried out in each of which two animals were injected intraperitoneally with a 10 per cent solution of an enzymatic casein hydrolysate, while one animal was simply pierced with a needle. For convenience, the latter procedure will be called "dry needle."

The liver extracts were prepared by excising approximately 3 gm. (weighed to the nearest 0.1 mg.) of tissue from the animals (killed by decapitation) and by homogenizing the tissue with 6 ml. of phosphate buffer (pH 7.35) for 5 minutes. The buffer employed contained NaCl (0.154 m), MgSO₄·7H₂O (0.154 m), and KCl (0.154 m) and was adjusted to pH 7.35 with HCl and Na₂HPO₄. After homogenization, the minced tissue mixture was centrifuged at about 8000 R.P.M. for 10 minutes. The supernatant was decanted and 2 ml. portions were used for the enzyme activity determination. The kidney was processed in a similar manner and ap-

^{*} A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists at Detroit, Michigan, April 18 to 22, 1949.

¹ Amigen, prepared by Mead Johnson and Company, Evansville, Indiana. We wish to express our appreciation to Dr. Warren M. Cox of that company for generously supplying us with this preparation.

proximately 1.2 gm. of tissue were homogenized with 10 ml. of the same buffer for 10 minutes. According to the findings of Blanchard *et al.* (1) most of the p enzyme probably would be inactivated by the processing procedure. Dry weight determinations were made by drying the tissue at 110° for 3 hours so that corrections could be made for variation in water content of the tissue.

It was necessary to attain a constancy of the tissue processing conditions so that the enzyme activity would vary only with the animal and its experimental environment. Accordingly, 25 and 45 minutes were allotted from the time the animals were killed until the tissue was homogenized, and from homogenization until the first readings were taken, respectively.

Since the effect of amino acid administration on AAO activity was to be determined after periods $\frac{1}{2}$ and $1\frac{1}{2}$ hours from the time of injection, animals were sacrificed at these time intervals and their tissues processed as related above.

In the Warburg manometric technique, employed for the determination of oxygen uptake, the flasks were filled as follows: main chamber, 2 ml. of buffered supernatant homogenate; side arm, experimental flask 0.2 ml. of a 0.1 m dl-alanine solution in buffer, control flask 0.2 ml. of buffer; center well, 0.2 ml. of a 10 per cent sodium hydroxide solution. After replacing the air in the flasks with oxygen, they were placed in a water bath and equilibrated for 10 minutes at 36.8°.

After tipping the substrate into the main chamber, readings were taken every 15 minutes for 1 hour while the flasks were shaken 80 times per minute. The oxygen uptake of the extracted homogenate is expressed in microliters of O_2 per gin. of wet tissue.

Experiments in vitro and in vivo have been carried out in a similar manner in order to determine the effects of insulin, adrenal cortical extract, and epinephrine on the AAO system.

Experiments in Vivo -(a) 0.5 i.u. of crystalline insulin (Squibb) was injected intramuscularly into the experimental animal 1 hour prior to decapitation; (b) 1 ml. of aqueous adrenal cortical extract (Upjohn) was injected intramuscularly hourly during a 4 hour period prior to sacrificing; and (c) 0.3 ml. of a 1:1000 adrenalin hydrochloride solution (Parke, Davis) was injected in the same manner as (b).

Experiments in Vitro 0.05 i.u. of insulin, 0.1 ml. of aqueous adrenal cortical extract (Upjohn), or 0.1 ml. of 1:25,000 adrenalin hydrochloride solution (Parke, Davis) was used in each of the control and the experimental flasks which were otherwise prepared as described previously.

² The control animals in this group were injected with the respective quantity of 10 per cent ethyl alcohol since the cortical extract is a 10 per cent alcoholic solution.

Control determinations were run for each group of the experiments in vitro and in vivo.

Blood glucose (2), urea (3), amino acids (4), and hematocrit determinations were carried out simultaneously on blood obtained at the time the animals were killed by decapitation.

The adrenalectomized animals were kept for 7 days on the Purina diet but given 1 per cent NaCl solution for drinking water. They then were taken off NaCl solution, given plain water for 3 days preceding the experiment, and fasted for the last 18 hours. The hypophysectomized animals were kept for 14 to 21 days on a milk, chopped meat, and bread diet before the experiment. This group had its own control group fed a similar diet. The thyroidectomized animals were maintained for at least 21 days before being subjected to experimental procedures. Only those animals that evinced a minimum decrease in basal metabolic rate of 25 per cent were used.

Sham operations (neck dissection) were performed on another group of animals in order to determine whether any effect on the AAO resulted from the operation alone. No changes were found when the results were compared with those of intact control animals.

RESULTS AND DISCUSSION

Liver Amino Acid Oxidase—From Table I it is obvious that administration of a casein hydrolysate to normal animals caused a pronounced increase in the liver AAO activity. However, neither adrenalectomized nor hypophysectomized animals similarly treated showed this increase. Therefore, the effect of the increased blood amino acid level on the liver AAO is probably mediated by the pituitary-adrenal system. The question whether the elevated blood amino acid level causes a direct or indirect stimulation of the anterior pituitary cannot as yet be answered.

The effect of the adrenal cortical secretion on the oxidase activity of the liver was also observed by experiments in vitro and in vivo. Table II illustrates that normal as well as adrenalectomized animals, given adrenal cortical extracts, showed an increased oxidase activity of the liver, whereas untreated adrenalectomized animals showed a decreased AAO activity (Table III). The experiments in vitro also demonstrated the accelerating effect of cortical extracts upon AAO activity of the liver (Table II).

The AAO activity of the liver of hypophysectomized animals was increased 100 per cent over that of normal animals (Table IV). This increase in activity was probably due to the absence of the pituitary growth factor and is in agreement with the concept that the growth hormone may inhibit amino acid catabolism. It has been shown by Szego and White (5) that growth hormone produces increased fatty acid metabolism

and fat deposition in the liver when administered to normal fasted animals. These investigators suggested that the growth hormone may either inhibit amino acid catabolism or accelerate fat metabolism. Our observations support the former postulate, for in our experiments the hypo-

TABLE I

Increase in Amino Acid Oxidase Activity after Amino Acid Injection

The values equal microliters and per cent increase in microliters of oxygen uptake of extract of tissue homogenate per gm. of tissue. Data computed from Tables III and IV.

Tissue	Time after injection	No	rmal	Adre	nalec- ized		hysec- ized		oidec- ized
	min.	μΙ.	per cent	μl.	per cent	μl.	per cent	μl.	per cent
Liver	30	14	50	-5	-17			9	33
	90	15	38	1	4	-31	-38	3	11
Kidney	30	119	89	-34	-13			-43	-14
	90	18	7	107	5 9	66	29	-93	-27

Table II

Effect of Hormones on Amino Acid Oxidase of Liver In Vitro and In Vivo

Oxygen uptake of extract of tissue homogenate given in microliters per gm. of tissue. The figures in parentheses represent the number of animals. In the experiments in vivo, the control animals of the adrenal cortical extract group were injected with either 10 per cent ethyl alcohol (see the text, foot-note 2) or the dry needle and those of the adrenalin and insulin groups with either 1 per cent NaCl solu-

tion or the dry needle as in the experimental animals.

ctivity
(14)
(2)
5 (2)
(14)
5 (20)
-19

physectomized animals showed an increased AAO activity in the liver. Russell and Cappiello (6) recently reported that the rate of urea formation was reduced after the administration of anterior pituitary growth hormone.

TABLE III

Total Respiration and Amino Acid Oxidase Activity of Experimental Animals Given 5 Ml. of Casein Hydrolysate Intraperitoneally

Oxygen uptake of extract of tissue homogenate in microliters per gm. of tissue. The figures in parentheses represent the number of animals.

Tissue	Time after		N	ormal	Adrenalectomized		
1 issue	injection			Buffer	Alanine	Oxidase activity	
	min.						
Liver	30	226	267	$41 \pm 4.5 (20)$	225	249	$24 \pm 4.3 (19)$
	90	224	279	$55 \pm 3.1 (21)$	179	207	$28 \pm 4.0 (7)$
Kidney	30	205	459	254 ± 36 (19)	169	400	231 ± 25 (14)
	90	207	477	270 ± 31 (28)	187	476	289 ± 20 (7)
			Hypoph	ysectomized	A	Thyroic	dectomized
Liver	30				219	246	$27 \pm 3.0 (8)$
	90	200	250	$50 \pm 5.7 (5)$	239	269	$30 \pm 4.3 (8)$
Kidney	30				195	463	268 ± 22 (8)
_	90	173	470	297 ± 30 (9)	194	451	$257 \pm 5 (8)$

TABLE IV

Total Respiration and Amino Acid Oxidase Activity of Control Animals Given Dry
Needle

Oxygen uptake of extract of tissue homogenate in microliters per gm. of tissue. The figures in parentheses represent the number of animals.

Tissue	Time after dry		N	ormal	Adrenalectomized			
1 issue	needle	Buffer	Alanine	Oxidase activity	Buffer	Alanine	Oxidase activity	
	min.							
Liver	0	211	270	$59 \pm 8 (10)$				
	30	208	235	$27 \pm 3.3 (8)$	197	226	$29 \pm 6.1 (9)$	
	90	235	275	$40 \pm 4.4 (9)$	171	198	$27 \pm 5.1 (5)$	
Kidney	30	206	341	$135 \pm 13.5 (6)$	197	462	265 ± 17 (6)	
•	90	220	472	252 ± 21 (13)	198	380	182 ± 14 (4)	
			Hypoph	ysectomized	Thyroidectomized			
Liver	0							
	30		ĺ		205	223	$18 \pm 2.0 (4)$	
·~(90	204	285	$81 \pm 9 (5)$	201	228	$27 \pm 3.1 (4)$	
ney	30				164	475	311 ± 18 (4)	
,	90	181	412	231 ± 15 (5)	201	551	$350 \pm 21 (4)$	

In thyroidectomized animals, administration of amino acids resulted in an increase in AAO activity of the liver which, however, was not as pronounced as in normal animals (Table I). According to Deane and Greep (7), thyroidectomy leads to an atrophy of the adrenal cortex. This may explain why the increase in liver AAO activity in thyroidectomized animals was not as great as in normal animals. Thyroidectomized control animals showed a decreased liver AAO activity (Table IV), a finding which is in agreement with that of Klein (8).

Kidney Amino Acid Oxidase—Comparable data were not obtained for the kidney AAO after amino acid administration (Table I). Results for normal kidney showed an 89 per cent increase in oxidase activity after ½ hour but only a negligible increase after 1½ hours. A similar type of disagreement was also observed in the kidney AAO activity of adrenalectomized and hypophysectomized animals. Although there was no increase after ½ hour in the adrenalectomized animals, there was a 59 per cent increase in the AAO activity after 1½ hours. Again, peculiarly, a 29 per cent increase in activity occurred in the kidney of hypophysectomized animals after 1½ hours. However, in the thyroidectomized animals, the kidney AAO activity was distinctly decreased after amino acid administration. This inhibitory effect still has to be elucidated.

There appears to be a distinction between the factors influencing AAO activity of the liver and of the kidney. The AAO activity in the kidney, apparently, is not only dependent upon endocrine factors but may also be directly influenced by the amino acid level of the blood. Thus, the initial increase in AAO activity of the normal animal's kidney $\frac{1}{2}$ hour after injection and the subsequent decrease after $1\frac{1}{2}$ hours (Table I) may be attributed to the corresponding rise and fall of the amino acid level of the blood. This view may similarly apply to the adrenalectomized and hypophysectomized groups where the findings were reversed; *i.e.*, the greater increase in the AAO activity occurred after $1\frac{1}{2}$ hours. In these instances, since the liver oxidase activity, in the absence of the activating mechanism of the pituitary-adrenal cortex system, could no longer participate normally in lowering the amino acid level, the effect of the gradual blood amino acid elevation was manifested in the kidney but not until a period of time had elapsed.

Normal and thyroidectomized animals, which were pierced with a dry needle $\frac{1}{2}$ hour previous to being sacrificed, showed a decreased AAO activity in the liver and kidney compared with animals sacrificed after $1\frac{1}{2}$ hours (Table IV). Since this decrease was not manifested in adrenalectomized animals similarly treated, and since it is associated with increased blood glucose levels in the normal and thyroidectomized control animals (Table VI), one may suspect that the secretion from the adrenal medulla may be responsible for the transient decrease in AAO activity. The results of experiments with epinephrine both *in vitro* and *in vivo* support this possibility (Table II). When a non-commercial epinephrine solution con-

taining crystalline epinephrine was used, the inhibitory effect lasted for 15 to 20 minutes only. This difference in effect may have been due to the presence of antioxidants in the commercial solutions.

It is also conceivable that the above mentioned inhibition may be due to the action of insulin on the AAO. The increased blood sugar levels caused by an epinephrine reaction may result in an increased insulin secretion. Although certain investigators (9, 10) have been able to show insulin inhibition of this enzyme, it was not possible to demonstrate this effect clearly by our technique, either *in vitro* or *in vivo* (Table II).

Blood Amino Acid, Glucose, Urea, and Hematocrit (Tables V, VI)—AAO activity may be correlated with the level of amino acid nitrogen, urea

TABLE V

Changes in Blood Constituents during Experimental Procedures

Hematocrit readings in per cent; amino acids in mg. per cent of amino acid N.

The figures in parentheses represent the number of animals.

Blood	Time after	No	ormal	Adrenalectomized			
constituents	injec- tion	Control	Injected	Control	Injected		
	min.						
Hemato-	30	52.5 ± 0.6 (4)	$55.4 \pm 1.0 (8)$	55.5 ± 1.3 (3)	60.0 ± 1.4 (5)		
crit	90	52.4 ± 0.4 (4)	56.5 ± 1.5 (6)	55.5 ± 0.8 (3)	$63.0 \pm 2.0 (5)$		
Amino	30	$12.1 \pm 1.3 (5)$	$18.5 \pm 1.6 (8)$	12.6 ± 1.3 (7)	$20.5 \pm 1.3 (13)$		
acids	90	$12.9 \pm 1.4 (9)$	$15.4 \pm 0.2 (12)$	14.5 ± 0.6 (3)	$22.3 \pm 1.8 (5)$		
		Hypophy	ysectomized	Thyroic	lectomized		
Hemato-	30			49.4 ± 1.5 (4)	51.2 ± 0.8 (8)		
crit	90			50.5 ± 0.9 (3)	$55.0 \pm 2.0 (6)$		
Amino	30			11.9 ± 0.9 (3)	$21.7 \pm 3.1 (6)$		
acids	90	12.5 ± 0.5 (4)	$16.0 \pm 1.2 (7)$	12.2 ± 0.4 (4)	16.0 ± 1.6 (8)		

nitrogen, and glucose in the blood. In normal animals (Table V), the amino acid nitrogen rose from 12.1 to 18.5 mg. per cent ½ hour after injection of the amino acid mixture and declined to 15.4 mg. per cent after 1½ hours, indicating rapid deamination. These variations in the blood amino acid nitrogen level can be linked with the respective changes in urea nitrogen and glucose levels of the blood (Table VI). While urea nitrogen was not greatly changed after ½ hour, it was elevated by 12.5 mg. per cent 1½ hours after the injection of amino acids. Similarly, in normal animals, there was no increase in blood glucose in ½ hour but a 10.8 mg. per cent increase occurred after 1½ hours. These results probably indicate that the increased deamination of amino acids leads to an increased formation of ammonia and, consequently, of urea and that,

simultaneously, increased gluconeogenesis takes place. Acceleration of these metabolic processes in normal animals had started in $\frac{1}{2}$ hour and was well established in $1\frac{1}{2}$ hours after the injection of the casein hydrolysate.

In the adrenalectomized animals, the increase in these transformations seemed to be slowed down or inhibited (Table V). These findings are in agreement with the generally accepted assumption that certain factors secreted by the adrenal cortex enhance protein catabolism.

The observation (Table VI) that there was a significant increase in urea formation in the adrenalectomized animals may seem surprising, since the

Table VI

Changes in Blood Constituents during Experimental Procedures

The values are given in mg. per cent—The figures in parentheses represent the number of arrivals.

Blood	Time after	Normal	Adrenalectomized
constituents	injec tion	Control Injected	Control Injected
	mın	_	
Urea as	30	¹ 18 9 ± 0 5 (+) 23 6 ± 1 8 (9)	$ 40.8 \pm 2.0 (3) 48.7 \pm 3.1 (4)$
urea N	90	$ 17\ 7\ \pm\ 0\ 3\ (5)\ 30\ 2\ \pm\ 0\ 8\ (8)$	$_{1}40\ 3\ \pm\ 2\ 6\ (3)\ 48\ 6\ \pm\ 2\ 8\ (2)$
Glucose	0	$^{1}68\ 2\ \pm\ 0\ 2\ (3)^{-1}$	
	30	$^{1}72\ 9\pm2\ 1\ (3)\ 66\ 6\pm2\ 4\ (8)$	$_{+}46\ 5\pm2\ 3\ (3)\ 36\ 0\pm6\ 1\ (5)$
	90	$166\ 0\ \pm\ 3\ 0\ (5)\ 76\ 8\ \pm\ 2\ 7\ (10)$	$ 47\ 8 \pm 2\ 0\ (3)\ 27\ 8 \pm 1\ 1\ (5)$
		Hypophysectomized	Thyroidectomized
Uica as	30		$28.1 \pm 0.9 \ (3) \ 31.7 \pm 1.8 \ (7)$
urea N	90	$46.8 \pm 2.2 \ (3) \ 59.4 \pm 1.6 \ (6)$	$29\ 1\pm 1\ 5\ (3)\ 39\ 3\pm 2\ 1\ (5)$
Glucose	0		$58.5 \pm 3.0 (3)$
	30		$ 73\ 7\pm 1\ 0\ (4) 77\ 6\pm 0\ 8\ (8)$
	90	$ 45\ 5\ \pm\ 1\ 5\ (2) $ $ 62\ 1\ \pm\ 3.6\ (4) $	$59\ 2\ \pm\ 2\ 5\ (4)\ 46\ 6\ \pm\ 3\ 1\ (8)$

lack of adrenal cortical secretion would lead one to believe that little or no deamination takes place. However, it must be recalled that the ability of the kidney to deaminate was not found to be as impaired as that of the liver in the adrenalectomized animals (Tables III, IV). Thus, ammonia formed in the kidneys of the animals which had been operated on may be utilized in the liver for the formation of urea. The impaired excretory capacity of the kidneys in these animals may account for retention of some urea.

Administration of amino acids to adrenalectomized animals was accompanied by a decrease in blood glucose, whereas the normal animals showed a hyperglycemia after an initial decrease (Table VI).

In hypophysectomized animals, the amino acid nitrogen level (16.0 mg. per cent) 1½ hours after amino acid administration was the same as that found in normal animals similarly treated (Table V). The control animals in the hypophysectomized group had an amino acid nitrogen level comparable to the normal controls (12.5 mg. per cent). The ability of the hypophysectomized animals to deaminate injected amino acids at a rate comparable to that observed in the normal animal may be due to the absence of the pituitary growth factor, as noted previously. Furthermore, the absence of this factor may explain the apparently accelerated protein catabolism, as shown by the finding that blood urea nitrogen was increased from 46.8 to 59.4 mg. per cent and glucose from 45.5 to 62.1 mg. per cent during this period (Table VI).

By the same criteria, thyroidectomized animals manifest the ability, although somewhat retarded, to metabolize amino acids. From Table V, it may be observed that the lowering of the amino acid nitrogen level was not quite as prompt or as marked but eventually, after injection of amino acids, the amino acid nitrogen level decreased from 21.7 mg. per cent in $\frac{1}{2}$ hour to 16.0 mg. per cent in $\frac{1}{2}$ hours. Similarly, the urea nitrogen levels, although increased, do not quite attain the increase that the normal animals showed in $\frac{1}{2}$ and $1\frac{1}{2}$ hours. This finding supplements the previously mentioned phenomenon of decreased liver AAO activity in thyroidectomized animals. The blood glucose levels in these animals increased after $\frac{1}{2}$ hour (3.9 mg. per cent) but decreased greatly (13 mg. per cent) $1\frac{1}{2}$ hours after injection.

The higher concentration of the blood urea in thyroidectomized control rats compared with normal control animals (Table VI) is probably due to the increased renal AAO activity in these animals (Table IV). Apparently, thyroidectomy produces an elevated resultant catabolism of amino acids. Such a postulation is in agreement with the findings of Persike (11) and of Rupp, Paschkis, and Cantarow (12).

It can be noted from Table V that the hematocrit was increased 6 to 8 per cent in the injected normal animals and 8 to 12 per cent in the adrenal ectomized animals.

SUMMARY

- 1. Data have been presented relating the blood amino acid level and the secretions of the hypophysis, adrenal, and thyroid to the activity of amino acid oxidase in the liver and the kidney of rats.
- 2. Administration of a casein hydrolysate to normal rats produces an increase in the amino acid oxidase activity of the liver and kidney in these animals.
 - 3. Administration of a casein hydrolysate to adrenalectomized or hypo-

physectomized animals does not produce this increase in the amino acid oxidase activity of liver.

- 4. Adrenal cortical extract accelerates the activity of this enzyme in the liver in vitro and in vivo.
- 5. The pituitary-adrenal cortex system mediates the stimulus for the acceleration of amino acid oxidase activity of the liver observed after amino acid administration but the nature of the stimulus remains to be determined.
- 6. Livers of hypophysectomized animals show increased amino acid oxidase activity which may be due to the absence of the growth factor of the pituitary.
- 7. Thyroidectomized animals show a decreased amino acid oxidase activity of the liver but an increased activity of the kidney. Administration of amino acids stimulates the liver oxidase.
- 8. Epinephrine inhibits amino acid oxidase activity of the liver. The effect of insulin is doubtful under our experimental conditions.
- 9. The amino acid oxidase activity of the liver and the kidney may respond similarly to certain endocrine stimuli. However, it appears that the blood amino acid level may influence the kidney oxidase activity directly but not that of the liver.

BIBLIOGRAPHY

- Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., J. Biol. Chem., 155, 421 (1944).
- 2. Somogyi, M., J. Biol. Chem., 160, 69 (1945).
- 3. Van Slyke, D. D., and Cullen, G. E., J. Biol. Chem., 24, 117 (1916).
- 4. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., J. Biol. Chem., 149, 255 (1943).
- 5. Szego, C. M., and White, A., Endocrinology, 44, 150 (1949).
- 6. Russell, J. A., and Cappiello, M., Endocrinology, 44, 333 (1949).
- 7. Deane, H. W., and Greep, R. O., Endocrinology, 41, 243 (1947).
- 8. Klein, J. R., J. Biol. Chem., 128, 659 (1939).
- 9. Bach, S. J., and Holmes, E. G., Biochem. J., 31, 89 (1937).
- Stadie, W. C., Lukens, F. D. W., and Zapp, J. A., Jr., J. Biol. Chem., 132, 393 (1940).
- 11. Persike, E. C., Endocrinology, 42, 356, 363 (1948).
- 12. Rupp, J., Paschkis, K. E., and Cantarow, A., Endocrinology, 44, 449 (1949).

THE FATE OF URIC ACID IN MAN*

By WILLIAM GEREN, AARON BENDICH, OSCAR BODANSKY, AND GEORGE BOSWORTH BROWN

(From the Laboratories of the Sloan-Kettering Institute for Cancer Research, New York)

(Received for publication, July 15, 1949)

The traditional assumption that uric acid represents the final endproduct of nucleic acid purine metabolism in man has received rather tenuous support from balance studies (1, 2) and from the failure to detect uricase in human tissues (3, 4). An opportunity to study directly the fate of uric acid in man was afforded by the availability of isotopically labeled uric acid (5) and this has now been done with uric acid administered both orally and intravenously.

EXPERIMENTAL

Isolation of Uric Acid from Urine—It was found that uric acid in human urine could be quantitatively adsorbed on charcoal (Darco) and that it could be eluted with hot 0.1 N sodium hydroxide solution. In a typical experiment, 430 ml. of filtered human urine were mixed with about 4 gm. of charcoal and placed overnight in the refrigerator. The charcoal was collected by filtration and was eluted by boiling with 30 ml. and then with 10 ml. of 0.1 N NaOH. The filtrate was chilled and was then acidified to about pH 4 with glacial acetic acid, whereupon 100 to 150 mg. of crude uric acid were precipitated. The crude uric acid was purified by dissolving it in a minimum volume of boiling water to which a few drops of 1 N NaOH had been added. The resulting solution was decolorized with a small amount of charcoal, then neutralized with acetic acid and concentrated at the boiling point to a small volume. The crystals obtained after chilling were dissolved in a small volume of 0.1 N NaOH. decolorized again if necessary, and reprecipitated with glacial acetic acid. The last purification step was repeated. The identity and homogeneity of the product were confirmed by counter-current distribution (6). In the experiment cited, 38 mg. of uric acid were obtained. Analytical values varied from 32.9 to 33.9 per cent N, compared with a theoretical N content of 33.4 per cent.

^{*} The authors gratefully acknowledge the assistance of the Office of Naval Research, the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and the James Foundation of New York, Inc.

Presented in part before the meeting of the American Society of Biological Chemists at Detroit, May 1, 1949.

Isolation of Urea—Urea was isolated from urine as the dixanthydrol derivative (7). The extent to which the urea isolated by this procedure was contaminated with uric acid was determined. In three experiments the N¹⁵ content¹ of urea ranged from 0.006 to 0.008 atom per cent excess when isolated from urines to which isotopic uric acid had been added to bring the levels of N¹⁵ in the total uric acid from 0.55 to 0.76 atom per cent excess N¹⁵. Since the abundance of N¹⁵ in urea from normal urine with respect to the reference standard in use is, as indicated below, 0.005 atom per cent excess, the average contamination of the urea by uric acid was thus 0.003 atom per cent, which represents not over 0.5 per cent of uric acid nitrogen in the isolated urea samples. The N¹⁵ level in urea isolated from a sample of urine containing uric acid with an N¹⁶ content of only 0.07 atom per cent excess was undetectable.

Isolation of Ammonia—Urine samples were made alkaline and aerated into standard dilute hydrochloric acid.

Determination of Abundance of N¹⁵ in Uric acid, Ammonia, and Urea in Normal Urine—The abundance of N¹⁵ in the urea isolated from the urine of three normal individuals was compared with the tank nitrogen which was in use as the reference standard. A total of fifteen independent analyses, each in triplicate, on these samples of urea was made and found to range from 0.0034 to 0.0050 with a mean of 0.0044 and a standard deviation of ±0.0005 atom per cent excess N¹⁵. On another day a series of eight determinations gave a mean of 0.0048 atom per cent excess. The mean for both series was 0.0045 atom per cent. A number of determinations of the N15 in the total nitrogen, the ammonia, and the uric acid from normal urines were of about the same magnitude. Consequently, 0.005 atom per cent excess N15 has been subtracted from each value listed here to compensate for the difference in abundance of N¹⁵ in the reference standard and in biological nitrogen. The increased abundance of N15 (above that of the nitrogen of air) reported previously (8) is in agreement with the more precisely determined figure of 0.005 atom per cent given here.

The standard deviation from the mean for the abundance of N^{15} in the urea from urine was ± 0.0005 . However, these determinations were all made at the same time and a wider variation may be expected among samples run under varying conditions. Seventeen determinations made over a period of 11 months on a sample of guanine, containing 0.143 atom per cent excess N^{15} , showed a standard deviation of 0.0013 atom per cent excess. This figure represents the precision obtained from day to day

¹ Consolidated Nier mass spectrometer, model 21-201.

GEREN, BENDICH, BODANSKY, AND BROWN

and it was decided that only differences about 2 times this standard deviation, or 0.002 atom per cent, were to be considered significant.

Determination of Uric Acid in Normal Human Urine by Means of Isotope Dilution (9)—Three separate random pools of urine were collected from several normal male adults. Precisely weighed samples of about 40 mg. of uric acid containing 2.28 atom per cent excess N¹⁵ were dissolved in a few ml. of 0.1 n NaOH and were added to 450 ml. specimens of the filtered urines. Charcoal was added after thorough mixing and uric acid was isolated and purified in the usual manner. A similar analysis was carried out on urine from the individual who was the subject of the experiments reported here. Uric acid determinations by the Archibald method (10) were carried out on the urine samples to which no isotopic

Table I

Comparison of Isotope Dilution and Colorimetric Methods for Determination of
Uric Acid in Human Urine

Urine		N ¹⁵ content	N15 content	Uric acid in total sample			
Specimen No. (450 ml each)	Isotopic uric acid added	of added uric acid	of isolated uric acid	By isotope Dilution	By colori- metric deter- mination	Factor	
	mg.	atom per cent excess N16	atum per cent excess N16	mg.	mg		
	39.9	2.28	0.524	134	158	1.18	
	39.7	2.28	0.491	145	163	1.12	
	39.9	2 28	0.386	196	253	1.29	
	40.1	2 28	0.299	265	309	1.17	

uric acid had been added. The results listed in Table I indicate that the colorimetric method yields uric acid values about 16 per cent (average of Specimens 1, 2, and 4) higher than the true uric acid level in the urines. Accordingly, all of the quantitative uric acid values recorded here have been obtained by dividing the colorimetric value by the factor 1.16 to correct for the difference in the results obtained by the colorimetric (Archibald) determination and the isotope dilution method of determination.

Oral Administration of Uric Acid—An 88 kilo normal adult male, who followed a reasonably low purine diet, ingested 154 mg. of uric acid containing 16.0 atom per cent excess N¹⁵ (5) preceding a light meal. Complete 6 hour urine collections were made for the first 3 days and 12 hour collections for the next 2 days. The volumes were recorded and the urines were preserved under toluene and kept in the refrigerator. Since it was found that toluene, when present in large amounts, interfered with the

adsorption of uric acid by charcoal, the toluene layer was removed prior to the determination. Samples were removed and total N and N¹⁵ were determined after Kjeldahl digestion. Uric acid was determined colori-

TABLE II
Urinary Exerction of Nitrogenous Components

Day No.	Period No.	Volume	Total ni	trogen	Urea nitrogen		Uric acid nitrogen		Ammonia nitrogen	
		After or	al admin	istratio	on of urio	acid	(8. 22 m	g. N ¹⁵))	
		ml.	alom per cent excess N16	mg. N15	atom per cent excess N18	mg. N15	alom per cent excess N18	mg. N15	aiom per ceni excess N18	mg. N
1	1	200	0.005	0.19	0.000	0.00	0.163	0.29	-0.001	0.000
-	2	310	0.031	1.36	0.020	0.68	0.260	0.55	0.010	0.009
	3	△25	0.020	0.93	0.019	0.69	0.205	0.28	0.013	0.022
	4	380	0.019	0.78	0.019	0.63	0.162	0.25	0.009	0.015
2	5	379	0.016	0.43	0.015	0.43	0.140	0.17	0.006	0.008
	6	225	0.012	0.42	0.013	0.35	0.112	0.21	0.010	0.011
	7	460	0.009	0.32	0.006	0.19	0.091	0.09	0.007	0.010
	8	365	0.011	0.40	0.009	0.26	0.082	0.14	0.008	0.012
3	9	1055	0.008	0.56	0.007	0.40	0.060	0.15	0.007	0.019
	10	650	0.007	0.52	0.005	0.26	0.038	0.11	0.003	0.001
Total, mg		5.91 72		3.89 47		2.24 27		0.107 1.3		
	Afte	er intrav	enous ad	ministr	ation of	uric ac	id (8.3	3 mg.	N15)	
1	1	1050	0.037	2.87	-0.003		1.86	3.15	0.015	
l	2	600	0.023	1.73	0.000		1.19	1.45	0.001	
2	3	800	0.014	1.09	0.002		0.801	1.27	0.001	
	4	800	0.010	0.72	0.003		0.520	0.65	0.001	
3	5	1550	0.003	0.42	0.003		0.290	0.83	0.001	
4	6	1550	0.002	0.30	0.001	į	0.151	0.39	0.001	
	7	1930	0.002	0.30	0.001		0.073	0.21	0.000	
5	,	Total (3 days), mg								
	(3 days), mg		6.83		1	i	7.35	I	
	(3 days			6.83 82				7.35 88		
Total), % of		: 1				1		

metrically (10); urea was determined by the urease method and ammonia by aeration.

Intravenous Administration of Uric Acid—6 months after the first experiment, the same subject received a total of 156 mg. of labeled uric acid (16.0 atom per cent excess N¹⁵) as a 0.5 per cent sterile solution in 0.7 per

cent Li₂CO₃ in three intravenous injections of equal volume spaced 30 minutes apart. Total 12 hour urine specimens were collected for the first 2 days and complete 24 hour specimens thereafter for 3 days. The isolations and determinations were carried out as before.

Results

The values from both experiments are listed in Table II, and have been corrected for natural N¹⁵ abundance above that of the reference standard.

Of the total of 8.22 mg. of N¹⁵ orally ingested in the form of uric acid, 5.91 mg. or 72 per cent of the administered dose had been excreted within

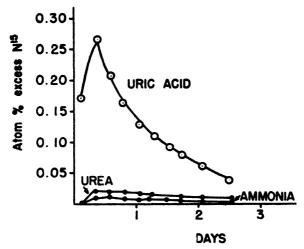


Fig. 1. Course of urinary excretion of N¹⁵ following oral administration of isotopic uric acid.

3 days. In terms of the various forms excreted, 2.24 mg. of N¹⁵ or only 27 per cent of the dose was recovered as unchanged uric acid, whereas 3.89 mg. of N¹⁵ or 47 per cent of the ingested uric acid had been degraded to urea. The N¹⁵ of the urinary ammonia amounted to but a small fraction, less than 2 per cent of the ingested uric acid N¹⁵. The course of excretion of the N¹⁵ is shown graphically in Fig. 1, and the total recoveries of N¹⁵ as uric acid and urea are presented in Fig. 2.

In the intravenous experiment, the total recovery from 8.33 mg. of injected uric acid N^{15} was 7.35 mg. of N^{15} as uric acid (88 per cent) after 3 days and 7.95 mg. of N^{15} (95 per cent) after 5 days. The course of the excretion of the N^{15} is plotted in Fig. 3 and the total recovery in Fig. 4.

The question arises as to whether there was any conversion of uric acid to urea following the intravenous injection of uric acid. This question

involves a consideration of the errors involved in the calculation of our values. The value for the mean difference between the N¹⁵ content of biological nitrogen and of the reference standard was 0.0045, s.d. ± 0.0005 , and a small variation of this mean value can lead to a difference in the correction factor of 0.001 atom per cent. In the case of the total nitrogen and the urea nitrogen many of the N¹⁵ values were very small. It was calculated that a constant difference of 0.001 in the correction factor applied amounts to a 5 per cent change in the total N¹⁵ excretion for these

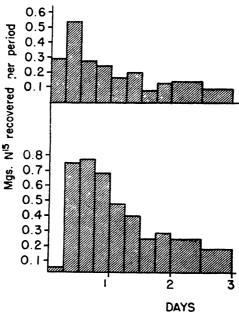


Fig. 2. Recovery of uric acid N¹⁵ (upper) and urea N¹⁵ (lower) following oral administration of isotopic uric acid.

components. Values for these components were therefore in error by ± 2.5 per cent. In addition, the variations possible in each determination of N^{1b} introduce another but smaller error which, calculated from the standard deviation of ± 0.0013 , amounts to from ± 0.7 to ± 2.3 per cent. In the case of the high values for the uric acid nitrogen these sources of error are insignificant, amounting to 0.3 and 0.1 per cent respectively in the two experiments.

In the intravenous experiment net values for the N¹⁵ in the urea (Table II) may be calculated to be 0.51 mg. or 6 ± 4 per cent of the administered dose in the first 3 days and 0.77 mg. or 9 ± 5 per cent in 5 days. In view of the magnitude of the errors involved in the very small N¹⁵ values from

which these were calculated, we do not feel that our results afford satisfactory evidence that there is a significant conversion of the intravenously administered uric acid to urea.

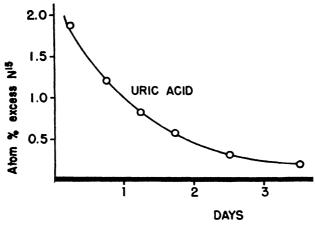


Fig. 3. Course of urinary excretion of N¹⁵ following intravenous administration of isotopic uric acid.

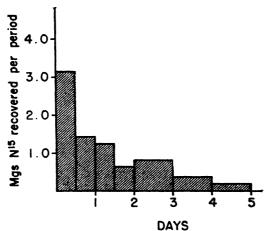


Fig. 4. Recovery of uric acid N¹⁵ following intravenous administration of isotopic uric acid.

Course of Excretion of Intravenously Administered Uric Acid—The simple assumption that the rate of excretion of intravenously injected uric acid is proportional to the amount of this substance present at any time leads to either of two equations (11). The first is

$$\frac{dx}{dt} = k(A - X) \tag{1}$$

in which A is equal to the amount of uric acid initially present expressed as mg. of N^{1b} excess, and X is equal to the amount of uric acid expressed as mg. of N^{1b} excess excreted at time t.

The second type of equation is

$$-\frac{dN}{dt} = KN \tag{2}$$

in which N is the concentration present at any time, t, in the uric acid, expressed as N^{15} atom per cent excess, and K is similarly a proportionality constant. The use of this equation does not necessitate a knowledge of the absolute amounts of N^{15} excreted but merely of its concentration in the uric acid nitrogen.

Table III

Conformance of Exerction of Uric Acid with Equation $k = 1/t \ln (A/(A-X))$

ı	x	A - X	$\frac{A}{A-X}$	k
days	mg. Nº18 excess	mg. N ¹⁸ excess		day
0	0	8.33		_
0.5	3.15	5.18	1.61	0.95
1.0	4.60	3.73	2.23	0.80
1.5	5.87	2.46	3.38	0.82
2.0	6.52	1.81	4.60	0.77

In order to see whether our data conform with the assumption made above, Equations 1 and 2 may be transformed into their usual integral forms.

$$k = \frac{1}{t} \ln \frac{A}{A - X} \tag{1'}$$

$$-\ln N + C = Kt \tag{2'}$$

Table III shows the results of substituting our data in Equation 1'. It may be seen that k has a fairly constant value for practically the entire course of excretion. The value of k for t = 0.5 day is somewhat high; only the last three values for k were averaged and yielded a value of k = 0.80 day $^{-1}$ and a value for the half life, $t_{0.5}$, for the injected uric acid of 0.87.

Equation 2' was also employed. It is apparent that the concentration of N¹⁵ in the uric acid of the urine is the same as that in the uric acid of the body. Hence, determination of the rate of change of concentration of N¹⁵ in the urinary uric acid represents the rate of change in the body. When natural logarithms of the values for the atom per cent excess N¹⁵

of the uric acid for the various periods were plotted against the mid-points of these periods, a straight line was obtained (Fig. 5). This result substantiated the assumption inherent in Equation 2'. The value of K was 0.83 day⁻¹ and the value for the half life $(t_0.5)$ was 0.83 day.

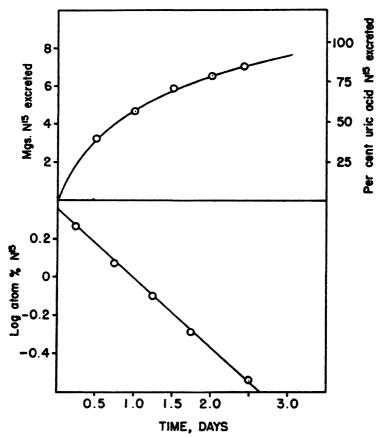


Fig. 5. Excretion of intravenously administered isotopic uric acid; upper curve, cumulative totals (cf. Equation 1'); lower curve, log atom per cent N¹⁵ (cf. Equation 2').

The value for the concentration of N^{15} at zero time, extrapolated from the plot of Fig. 5, was 2.28 atom per cent N^{15} . Since the concentration in the 156 mg. of uric acid which was injected was 16.0 atom per cent N^{15} , the body pool at zero time after injection was $156 \times 16.0/2.28 = 1090$ mg. of uric acid. When this was corrected for the injected uric acid, a value of 944 mg. was obtained for the total available uric acid in the individual.

A value of 4.6 mg. per cent of uric acid was found in the blood of the experimental subject immediately before the injection. From this and the total pool of available uric acid a "uric acid space" of about 20 liters for this individual may be calculated. Thus the available uric acid was present in a volume in accordance with the usual value for the extracellular space in an individual of this weight, 88 kilos.

DISCUSSION

In the case of the orally administered uric acid, the appreciable amount of N¹⁵ excreted in the urea of the urine indicated unequivocally that extensive degradation of the uric acid had resulted. However, in the case of the intravenously administered uric acid, essentially all of the administered isotopic nitrogen was recovered as urinary uric acid, and the excretion of N¹⁵ in the urea fractions was insignificant. These results support the contention that uric acid represents the final stage of the oxidation of purines in man and are in agreement with the reported absence of uricase in human tissues (3, 4).

In view of the lack of degradation of the intravenously administered uric acid, it must be concluded that the degradation of the orally administered uric acid is associated with its passage through the gut. It is most probable that intestinal bacteria are responsible (12), the products being absorbed from the gut and excreted in the urine. If this is the case, the variability in the recoveries of administered uric acid (1), purines (13), and nucleic acid derivatives (14) reported from time to time may be due to variations in the bacterial population in the individuals involved.

It is of interest to recall that urea did not arise from orally ingested uric acid in the rat (15), although there was very extensive conversion to allantoin. It may be that the intestinal flora in the two species are different, or that, in both species, the bacteria converted some uric acid to allantoin only. In this case the rat tissues would not degrade that allantoin further, while the human may be able to do so.

The possibility that ingested uric acid is converted in the gastrointestinal tract to some derivative, for instance to the riboside (16), must be admitted. Such a derivative might be oxidized to urea, whereas uric acid is not.

The authors wish to thank Roscoe C. Funk, Jr., John Deonarine, and Jean Birnbaum for assistance with the various analyses.

SUMMARY

It has been shown in man that orally administered uric acid is extensively degraded to urea, whereas intravenously administered uric acid is excreted essentially unchanged.

The pool of available uric acid in man has been shown to be somewhat less than 1 gm. and the half life, $t_{0.5}$, of this uric acid is less than 1 day (0.85 day).

A method for the isolation of uric acid and a comparison of the uric acid content of normal human urine, as determined colorimetrically and by the isotope dilution technique, have been presented.

BIBLIOGRAPHY

- 1. Folin, O., Berglund, H., and Derick, C., J. Biol. Chem., 60, 361 (1924).
- 2. Thannhauser, S. J., and Weinschenk, M., Deut. Arch. klin. Med., 139, 100 (1922).
- 3. Wiechowski, W., Beitr. chem. Physiol. u. Path., 9, 295 (1907); 11, 109 (1908).
- 4. Enzo, L., Boll. Soc. ital. biol. sper., 22, 556 (1946).
- Cavalieri, L. F., Blair, V. E., and Brown, G. B., J. Am. Chem. Soc., 70, 1240 (1948).
- 6. Tinker, J. F., and Brown, G. B., J. Biol. Chem., 173, 585 (1948).
- Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., J. Biol. Chem., 172, 469 (1948).
- 8. Schoenheimer, R., and Rittenberg, D., J. Biol. Chem., 127, 285 (1939).
- 9. Rittenberg, D., and Foster, G. L., J. Biol. Chem., 133, 737 (1940).
- Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., J. Clin. Endocrinol., 8, 15 (1948).
- 11. Clark, W. M., Topics in physical chemistry, Baltimore, 177 (1948).
- 12. Mitchell, N. B., and Levine, M., J. Bact., 36, 587 (1938).
- 13. Mendel, L. B., and Lyman, J. F., J. Biol. Chem., 8, 115 (1910-11).
- 14. Rose, W. C., Physiol. Rev., 3, 544 (1923).
- 15. Brown, G. B., Roll, P. M., and Cavalieri, L. F., J. Biol. Chem., 171, 835 (1947).
- 16. Newton, E. B., and Davis, A. R., J. Biol. Chem., 54, 603 (1922).

STUDIES ON THE UTILIZATION OF ACETIC ACID FOR THE BIOLOGICAL SYNTHESIS OF CHOLESTEROL*†

BY HENRY N. LITTLE AND KONRAD BLOCH

(From the Department of Biochemistry and the Institute of Radiobiology and Biophysics,
University of Chicago, Chicago)

(Received for publication, July 29, 1949)

Previous work (1, 2) has demonstrated that acetic acid is an important source of carbon and hydrogen in the total synthesis of steroids by the animal body, and that this compound is superior in this respect to various other organic molecules. Experiments with deuterioacetate led to the conclusion that 2-carbon compounds participate in the synthesis of both the nucleus and of the side chain of cholesterol and that, of the carbon and hydrogen atoms in the steroid molecule, at least half is derived from those of acetate. The present investigation was undertaken in order to examine the rôle of acetic acid in greater detail. To this end cholesterol synthesis has been studied with the aid of acetic acid labeled in either the carboxyl or methyl group, or acetic acid labeled by C13 as well as C¹⁴. In this way the relative utilization of the 2 carbon atoms of acetic acid for the synthesis of cholesterol as a whole, as well as for the aliphatic and cyclic moieties of the molecule, could be determined. In a few cases the source of individual carbon atoms of cholesterol has been identified. By degradation of isotopic cholesterol the origin of the isopropyl group in the sterol side chain, of the angular methyl groups, and of 2 carbon atoms in the ring system has been established.

The labeled cholesterol used in the present experiments was obtained by synthesis *in vitro* in surviving rat liver (3). Under these conditions the isotopic substrate is utilized with greater economy than in intact animals. In order to secure the relatively large quantities of steroid which were necessary for degradation studies, the biosynthesis was carried out with acetate labeled by C¹⁴ of high specific activity. The resulting cholesterol could then be diluted considerably by normal material.

DISCUSSION

Although the earlier work with deuterioacetate and with acetic acid labeled in the carboxyl group has shown qualitatively that both carbon atoms

^{*} Presented in part at the meeting of the American Society of Biological Chemists at Detroit, April, 1949.

[†] Supported by a grant-in-aid from the Life Insurance Medical Research Fund and by the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

of this compound participate in cholesterol formation, it was unknown whether they were utilized equally in the synthetic process. Since cholesterol has an odd number of carbon atoms, it is clear that, unless other precursors exist, the carboxyl and methyl carbon atoms of acetic acid cannot appear in the sterol molecule in equal numbers. Also, the presence in cholesterol of branched structures, such as the isopropyl group of the aliphatic chain, suggests that decarboxylations may occur at an intermediate stage of the synthesis. To what extent the 2 carbon atoms of acetic acid are utilized may be determined with the aid of acetic acid in which 1 of the carbon atoms is labeled by C¹³ and the other by C¹⁴. The ratio of the 2

Table I
Utilization of Methyl and Carboxyl Carbon Atoms of Acetic Acid for Cholesterol
Synthesis

magners decorderate and extendibilities are the	Cholesterol					
Precursor	C13	C14		sotope con- on* due to	Methyl carbon Carboxyl carbon	
		Č	Methyl carbon	Carboxyl carbon		
	atom per cent excess	counts per min	_	and their		
Experiment I C'13H3C'14O()II C'14, 9 26 atom (cercess, C'14,	0 200	426	2 16	1 61	1 32	
26,100 c p m Experiment II (14H ₃ C ¹³ OOH C ¹³ , 9 9 atom ' excess, C ¹⁴ , 105,000 c p m	0 307	3900	3 78	3 09	1 22	

^{*} Relative isotope concentration = $\frac{\text{atom } {}^{C}_{/O} \text{ excess } C^{13} \text{ or counts per min } C^{14} \text{ in cholesterol}}{\text{atom } {}^{C}_{/O} \text{ excess } C^{13} \text{ or counts per min } C^{14} \text{ in precursor}}$

carbon isotopes in cholesterol compared to the isotope ratio in the precursor will indicate the relative proportion of methyl and carboxyl carbon atoms of acetic acid in the synthetic product. The data in Table I indicate that the ratio of methyl carbon to carboxyl carbon in cholesterol is significantly greater than 1 and hence that methyl carbons predominate. If it is assumed that all carbon atoms of the sterol are furnished by acetate, then the observed ratio of 1.27, the average value of Experiments I and II, indicates that of the total of 27 carbon atoms in the sterol molecule 15 are derived from methyl groups and 12 from carboxyl groups, respectively, of acetic acid. This deduction will be valid only if acetic acid is the sole carbon source for cholesterol and if a given carbon atom of cholesterol is derived from either a carboxyl or methyl carbon atom of acetic acid but

not from both. Furthermore the carbon atoms of acetic acid which serve as the immediate source should have the same isotope concentration regardless of their subsequent location in cholesterol. As is shown later, the isotope concentrations observed for individual carbon atoms of cholesterol are on the whole consistent with these assumptions.

The calculation of the methyl carbon to carboxyl carbon ratio required four isotope analyses. Since the error of each determination is 5 per cent, the probable error of this ratio is about 10 per cent. Nevertheless, the close agreement of the data from two different experiments suggests that the ratio of 1.27 has real significance.\(^1\) An independent check of the accuracy of the analytical values is provided by the isotope ratios in the saturated fatty acids which were isolated from the same experiments. In accord with previous findings, which indicated that the higher fatty acids are formed by multiple condensation of 2-carbon units (4), the ratio of methyl carbon to carboxyl carbon in the saturated fatty acids in two experiments was found to be 0.98 and 1.08.

The value of 1.27 for the ratio of methyl carbon to carboxyl carbon shows that in the course of cholesterol formation from acetate decarboxylations take place. There is no evidence to indicate at which stage of the condensation process carboxyl groups are removed. However, it may be mentioned here that, as shown later in this report, the isopropyl portion of the cholesterol side chain is composed of two methyl groups and one carboxyl group of acetic acid, and hence may result from the condensation of 2 molecules of acetate to a 4-carbon compound and its subsequent decarboxylation to a 3-carbon unit.

The participation of acetic acid in the synthesis of both the isooctyl and cyclic moieties of cholesterol had been established with the aid of deuterium as a tracer (1). The quantitative significance of the results obtained on thermolysis of deuteriocholesteryl chloride was open to question, since the drastic conditions necessary for the splitting of the cholesterol molecule could have resulted in intramolecular hydrogen shifts. We have therefore repeated the degradation with cholesterol derived from C¹⁴-acetate and have determined the C¹⁴ content of the isooctane-isooctene mixture and of the hydrocarbon C₁₉H₂₀, fractions which represent the side chain and the nucleus of cholesterol. The results obtained (Table II) with cholesterol synthesized from carbon-labeled acetic acid confirm our earlier conclusion that the carbon atoms of acetic acid are utilized in the formation of both the aliphatic and the cyclic portion of cholesterol. The isotope concentration of the nucleus compared to that of the side chain was found to depend on the position of the label in the precursor. In the case of

¹ If the ratio methyl carbon to carboxyl carbon were 16:11 or 14:13, the isotope ratios should be 1.45 and 1.08 respectively.

cholesterol derived from deuterioacetate, the lower isotope concentration in the nucleus may merely be due to the fact that hydrogen bound to methyl groups accounts for more than half of all the hydrogen atoms in the isooctyl side chain but only for 20 per cent of all the hydrogen atoms in the ring structure. It may be assumed that, during the condensation process, the deuterium in those methyl groups of acetic acid which still appear as methyl groups in cholesterol is less likely to be lost or replaced by ordinary hydrogen than the deuterium of methyl groups which become part of the ring structure. The isotope concentrations in the two portions of the sterol did not differ significantly when acetic acid labeled by C¹⁴ in the methyl group was the precursor. On the basis of these data methyl groups of acetic acid appear to supply approximately the same fraction of the carbon atoms in the side chain and in the nucleus of cholesterol. On

Table II
Incorporation of Labeled Acetic Acid into Cholesterol and Degradation Products

	Atom per o	Atom per cent excess D or counts per min C14 in			
Precursor	Cholesterol	Nucleus (C18Ha0) (A)	Isooctyl side chain (B)	(A) (B)	
Deuterioacetic acid*	0.21	0.18	0.26	0.69	
Acetic acid-2-C14	71	76	74	1.03	
" "		400	424	0.94	
" acid-1-C14	93	98	72	1.36	
« «	39	45	36	1.25	

^{*} Data taken from Bloch and Rittenberg (1).

the other hand, in cholesterol formed from CH₃Cl⁴OOH, the radioactivity was significantly higher in the nucleus, indicating that the carboxyl group of acetic acid contributes a greater share of the ring carbon atoms than of those in the side chain. The observation that the isotope distribution in the two moieties of cholesterol depends on the position of the label in the acetic acid used could not be explained readily if the 2 carbon atoms of acetic acid participated equally in cholesterol synthesis. However, it is consistent with the above finding that a greater number of methyl than carboxyl carbon atoms of acetic acid is employed in steroid synthesis. From the data in Tables I and II, it follows that the ratio of methyl carbon to carboxyl carbon, which is 1.27 for the total cholesterol molecule, must be greater than this value for the side chain and smaller than 1.27 in the cholesterol nucleus. The ratios which are in nearest agreement with the experimental data are 5:3 or 1.67 for the isooctyl side chain and 10:9 or 1.1 for the cholesterol nucleus. On the basis of this distribution of

methyl and carboxyl carbons in the nucleus and side chain, the calculated ratio (A):(B) for acetic acid-2- \mathbb{C}^{14} is 0.84 and for acetic acid-1- \mathbb{C}^{14} 1.26. Isotope analyses of greater precision are needed to establish more completely the accuracy of these ratios.

Isotope Concentration of Individual Carbon Atoms in Cholesterol—By isotope analysis of the angular methyl groups and of the isopropyl moiety of the isopropyl side chain, the origin of some individual carbon atoms of the cholesterol molecule has been determined. The choice of these carbon atoms was prompted by the relative ease of their isolation and by our

Table III

Distribution of C¹⁴ in Isopropyl Group of Cholesterol Side Chain

		Cholesterol formed from			
Compound analyzed	CH ₁	C1400H	C14H4COOH		
	Found	Calculated	Found	Calculated	
	c.þ.m.	c.p.m.	c.p.m.	c.p.m.	
Cholestane	93		71		
Acetone	62	70*†	72	85*§	
Iodoform (C ₂₆ , C ₂₇)	20	0†	100	1285	
Carbonyl carbon of acetone (C26)	146‡	209†	16‡	0§	

^{*} Calculated on the assumption that the methyl and carboxyl carbon atoms of acetic acid are present in cholesterol in a ratio of 15:12.

interest in the biological synthesis of branched chain structures. Cholesterol synthesized from acetic acid-1-C¹⁴ and from acetic acid-2-C¹⁴ was used for this purpose.

The formation of acetone and allocholanic acid on chromic acid oxidation of cholestane (5) constitutes the classical proof for the presence of a terminal isopropyl group in the aliphatic side chain of cholesterol. Hence, we have assumed that the acetone obtained by oxidation of cholestane is derived exclusively from carbon atoms 25, 26, and 27. The isotope concentrations were determined in acetone and in the iodoform derived from it, which represents C_{26} and C_{27} . The isotope content of the carbonyl atom of acetone (C_{2b}) was calculated by difference. It is clear from the data in Table III that acetic acid had been utilized in the synthesis of the isopro-

[†] Calculated from the C¹⁴ content of cholestane for 1 isotopic carbon (C₂₈) per molecule of acetone.

t Calculated from the observed values for acetone and iodoform.

[§] Calculated from the C^{14} content of cholestane for 2 isotopic carbon atoms (C_{26}, C_{27}) per molecule of acetone. The predicted value of iodoform is 108 if the C^{14} concentration of C_{26} and C_{27} is calculated from the observed value for acetone (72 counts) from which the iodoform was obtained.

pyl group of cholesterol. When methyl-labeled acetic acid was the precursor of the steroid, the C¹⁴ content of the iodoform fraction (C₂₆ and C₂₇) accounted for nearly all the C¹⁴ in the acetone. On the other hand, in cholesterol synthesized from carboxyl-labeled acetic acid, the methyl group of acetone had a very low radioactivity, showing that in this case the carbonyl group of acetone (C₂₅) contained most of the isotope. Carbon atoms 26 and 27 of cholesterol therefore originate from the methyl groups of acetic acid, while carbon atom 25 is derived from the carboxyl carbon of acetate (Fig. 1). It should be pointed out that in both experiments the C¹⁴ content of the "labeled" carbon atoms was about 15 to 20 per cent lower than was expected from the isotope concentration of the steroid and that those positions which one would expect to be unlabeled contained a small but significant radioactivity. These discrepancies may be attrib-

uted either to shortcomings of the degradative and analytical procedures or perhaps to a biological redistribution of labeled carbon during the biosynthesis. It is worthy of note that the formation of the isopropyl group in the side chain of cholesterol from the elements of acetic acid is the only known instance of the synthesis of a branched chain structure in the animal organism. All other constituents of the animal body which have branched carbon chains are either essential amino acids or vitamins.

Origin of Carbon Atoms 10, 17, 18, and 19—In the oxidation of organic compounds by a mixture of chromic and sulfuric acids, methyl groups and their adjoining carbon atoms are converted to acetic acid. This procedure has been made the basis for an analytical determination of carbon-bound methyl groups (6). We have applied this procedure to the nuclear hydrocarbon C₁₀H₃₀ which is obtained by thermolysis of cholesteryl chloride. If it is assumed that this hydrocarbon has the structural formula proposed by Bergmann and Bergmann (7), chromic acid oxidation should yield a maximum of 2 moles of acetic acid (degradation)² from the methyl carbon atoms 18 and 19 and their adjoining carbon atoms (C₁₀ and C₁₇) (Fig. 2). The observed yield of acetic acid (degradation) was 1.3 to 1.5 moles. The acid was found to contain isotopic carbon both when methyl-

² The acetic acid resulting from the degradation is designated as acetic acid (degradation) in order to distinguish it from the acetic acid which serves as a precursor in steroid synthesis and which is designated acetic acid (precursor).

labeled and carboxyl-labeled acetic acid had been the cholesterol precursor. The acetic acid obtained on chromic acid oxidation of the cholesterol nucleus is derived from 4 carbon atoms of the steroid. Therefore, the isotope concentrations observed for the methyl groups of acetic acid (degradation) reflect the average C¹⁴ content of carbon atoms 18 and 19, and those given for the carboxyl group of acetic acid³ (degradation) represent the average

Table IV

Isotope Concentration in Angular Methyl Groups and C₁₀ and C₁₇ of Cholesterol

	Cholesterol synthesized from						
Compound analyzed	CH ₂ C ¹⁴ OOH C ¹⁴ H ₂ CO (Experiment I) (Experime						
	Found	Calcu- lation A*	Calcu- lation B*	Found	Calcu- lation A*	Calcu- lation B*	
	c.p.m.	c.p.m.	c.p.m.	с.р.т.	c.p.m.	c.p.m.	
1. Cholesterol	93			71			
2. Acetic acid by oxidation of C ₁₉ H ₃₀	64	105†	53†	76	64†	96†	
3. $CO_2(C_{10} + C_{17})$ from (2)	120	128	128	40	0	50	
4. Iodoform from methyl carbons $(C_{18} + C_{19})$ of (2)	2	0	0	105	152	101	

^{*} The two methods of calculation (A and B) are explained in the text.

concentration of carbon atoms 10 and 17 (Table IV). In cholesterol for which CH₈C¹⁴OOH had been the precursor, the angular methyl groups contained no C¹⁴. On the other hand, the isotope concentration was high at carbon atoms 18 and 19 in cholesterol which had been synthesized from C¹⁴H₃COOH, demonstrating that methyl groups of acetic acid are the source of the angular methyl groups of the steroid molecule. In Experiment I (Table IV), the isotope concentration of the acetic acid obtained

[†] Calculated on the assumption that the methyl and carboxyl carbon atoms of acetic acid are present in cholesterol in a ratio of 15:12. The calculated values in lines (3) and (4) are based on the observed C¹⁴ content of the acetic acid (line(2)).

³ It is assumed that the acetic acid (degradation) is formed in equal amounts from carbon atoms 18 and 10, and 19 and 17 respectively.

by degradation was entirely accounted for by the C14 content of its carboxyl group, and therefore either carbon atom 10 or 17, or both, must have been derived from carboxyl groups of the precursor acetic acid. It will be noted that in Experiment II (Table IV) the CO₂ fraction also had a significant radioactivity, which suggests that this fraction (C_{10} and C_{17}) had originated in part from methyl groups of acetic acid. It may further be seen that in Experiment I (Table IV) the acetic acid (degradation) had an appreciably lower isotope content and in Experiment II a higher isotope concentration than would have been expected if methyl groups of acetic acid (precursor) had been the source of carbon atoms 18 and 19 only, and if carboxyl groups of acetic acid had supplied both carbon atoms 10 and 17 (Calculation A). It should be recalled here that the methyl carbon 19 changes its position during the thermolysis of cholesteryl chloride and becomes attached to C_{17} (7). While one might expect both angular methyl groups in cholesterol to be attached to carbons which were originally carboxyl carbons of acetic acid, this need not be the case for C₁₀ after it has migrated to C₁₇. Since, as indicated in Experiment II (Table IV), the mixture of C₁₀ and C₁₇ contained a significant isotope concentration, 1 of these 2 carbon atoms may have been derived from methyl groups of acetic acid, and it seems likely that C₁₇ was the labeled one in this case. With these considerations in mind, we have calculated (Calculation B) the expected isotope concentrations of carbon atoms 10, 17, 18, and 19 and have assumed that, in Experiment I, of the 2 molecules of acetic acid formed on degradation, 1 contains no isotope and the other C¹⁴ in the carboxyl group (C₁₀), while in Experiment II 1 molecule of acetic acid is doubly labeled (C₁₇ and C₁₉) and the other singly (C₁₈). The experimental data for the most part are in satisfactory agreement with this assumption.

Admittedly the procedures which have been employed in order to determine the isotope concentration of the angular methyl groups are not unequivocal. We have therefore carried out an independent degradation experiment to substantiate the above results. When cholesterol is heated in the presence of palladium-charcoal, about 4 moles of hydrogen and 1 mole of methane are evolved per mole of cholesterol (8, 9). According to Ruzicka et al. (9), the aromatization of Rings A and B of cholesterol causes the elimination of the angular methyl group (C₁₈) in the form of methane. When this reaction was applied to cholesterol which had been synthesized from CH₃C¹⁴OOH, the methane produced had a low isotope concentration, but when cholesterol synthesized from C14H3COOH was subjected to dehydrogenation, the methane contained 73 to 81 per cent of the expected radioactivity (Table V). These results taken together with those observed on chromic acid oxidation of the cholesterol nucleus (C₁₉H₃₀) strongly indicate that the angular carbon atoms 18 and 19 have their origin in methyl groups of acetic acid. The source of carbon atoms 10

and 17 is less certain, although it appears from the analytical data that one of them is derived from a carboxyl and the other from a methyl group of acetic acid. We conclude tentatively that C_{10} is furnished by a carboxyl carbon of acetic acid and C_{17} by a methyl group.

Previous considerations of the quantitative significance of acetic acid as a sterol precursor were based on experiments with intact rats (1, 2). These animals received deuterioacetate or C¹⁸-acetate for a relatively short time, and it was computed that under these conditions acetate furnished about half of the carbon and hydrogen atoms of cholesterol. Since the calculation of these values included an estimate of the half life time of cholesterol and of the dilution of dietary isotopic acetate by acetic acid from endogenous sources, quantities which cannot be determined very accurately, the

TABLE V

Radioactivity of Methane (C₁₈) Formed by Dehydrogenation of Cholesterol

			Cholesterol synthesized from		
		-	CH ₂ C ¹⁴ OOH (Experiment I)	C14HsCOOH (Experiment II)	
	PF BRIDGE	The second section of the section of the section of the second section of the section of t	c.p.m.	c.p.m.	
Choleste	rol		92	59	
Methane	Samp	le 1*	13 (207)†	86 (106)†	
44	ı.	2	23	77	
"	" "	3	32	80	
"	"	4	43	83	

^{*} See "Experimental" for explanation of Samples 1 to 4.

values given were considered to be approximate only. The results reported here on the isotope concentration of individual carbon atoms of the steroid molecule furnish independent evidence that 2-carbon compounds are the principal source of carbon in the biosynthesis of cholesterol. If acetic acid or an equivalent 2-carbon compound was the sole precursor, then any single carbon atom in the sterol which is synthesized from acetic acid labeled in one position only should contain either about twice the isotope concentration of the total molecule or no excess isotope at all. On the other hand, if, for example, acetic acid supplied only half of all the carbon atoms, then any individual carbon of cholesterol should contain either

[†] The values in parentheses are the calculated isotope concentrations of a single carbon if derived from the labeled carbon of the precursor, corrected for the ratio of 15:12 for the ratio of methyl carbon to carboxyl carbon in cholesterol.

⁴ Since the methyl and carboxyl carbon atoms of acetic acid are not used equally in the synthetic process but in a ratio of 1.27, the isotope concentration of an individual carbon atom should be either 1.8 or 2.5 times that of the total steroid molecule.

about 4 times the isotope concentration of the total molecule or no excess isotope at all. Since the radioactivities at those carbon atoms which were analyzed individually were found to contain very little radioactivity when acetic acid labeled in one position was the precursor and contained close to the theoretical radioactivity in the case of the acetic acid labeled at the other carbon atom, it is probable that this will also be the case for the remainder of carbon atoms in the sterol molecule. The close agreement between postulated and observed isotope distribution is perhaps the most convincing evidence that a 2-carbon compound is the principal if not the sole building block of cholesterol.

EXPERIMENTAL

Preparation of Labeled Acetic Acid—Acetic acid-1-C¹⁴ was prepared from C¹⁴O₂ and methyl magnesium bromide.

Acetic acid-1- C^{13} was obtained from acetonitrile, as described by Weinhouse et al. (10).

Acetic Acid-2-C¹⁴—20 mm of methyl alcohol containing C¹⁴ were mixed with 15 ml. of hydriodic acid (sp. gr. 1.7) and a trace of red phosphorus, and heated under a reflux for 1 hour. A slow stream of nitrogen was passed through the reaction flask and the methyl iodide was collected in a trap cooled by dry ice. The methyl iodide (1.05 ml.) was transferred to a solution containing 1.01 gm. of sodium cyanide in 1.8 ml. of water and the mixture heated under a reflux for 6 hours. Acetonitrile was distilled from the reaction mixture with several additions of water to the reaction flask. The distillate containing the acetonitrile was hydrolyzed by heating under a reflux with 10 per cent sodium hydroxide for 24 hours. For purification the solution was heated for an additional half hour in the presence of potassium permanganate. Acetic acid was obtained by steam distillation of the acidified solution. The yield based on methyl alcohol was 66 per cent.

Acetic Acid-2-C¹³—C¹³-methyl iodide⁶ was allowed to react with KCN and converted to acetic acid as described for the preparation of acetic acid-2-C¹⁴.

Preparation of Labeled Cholesterol—The isotopic cholesterol used in the present experiments was obtained by incubation of rat liver slices in the presence of isotopic acetate as described before (3). Tissue from young male rats of the Sprague-Dawley strain (60 to 100 gm. in weight) was employed, since it had been noted (3) that the incorporation of isotopic carbon into cholesterol was considerably faster in younger than in older animals. In a typical experiment, 1.5 gm. of liver slices were incubated in 12 ml. of Krebs' phosphate buffer, pH 7.4, which contained 3 mg. of appro-

This procedure was worked out by I. Zabin in this laboratory.

[•] Furnished as a contribution by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

priately labeled acetic acid. The flasks were shaken at 37° for 6 hours. The gas phase was pure oxygen which was replaced every 2 hours. Cholesterol was isolated by way of cholesterol digitonide in the usual manner. Under our experimental conditions, the isotope concentration or specific activity in the isolated cholesterol varied from 2 to 4 per cent of that of the acetate used. This permitted a several hundred fold dilution of the C¹⁴-containing sterol by non-isotopic cholesterol, thus providing adequate amounts of marked sterol for degradation studies. In the experiments in which the utilization of the 2 carbon atoms of acetic acid for cholesterol synthesis was determined, appropriate mixtures of the differently labeled acetates were added to the incubation medium. Their isotope concentrations are given in Tables I to VI.

Degradation of Cholesterol into Nucleus and Side Chain—Cholesteryl chloride prepared from cholesterol was pyrolyzed as described before (1). It was assumed that the low boiling fraction was a mixture of isooctane and isooctene and represented the isooctyl side chain of cholesterol. This fraction was analyzed for isotope content after redistillation. The material remaining after removal of the volatile hydrocarbon was distilled in vacuo and yielded as the main fraction a hydrocarbon distilling at 185–230° at 1 mm. of Hg. The elementary composition and optical rotation of this material corresponded to those for the hydrocarbon C₁₉H₃₀ described by Bergmann and Bergmann (7).

C₁₉H₂₀. Calculated, C 88.4, H 11.6; found, C 88.3, H 11.7

The hydrocarbon obtained from two different runs had $[\alpha]_{\rm p}^{25} = +30.3^{\circ}$ and $+33.5^{\circ}$ (2 per cent in benzene) respectively. This hydrocarbon represents the intact steroid skeleton and is referred to in this paper as the cholesterol nucleus.

Oxidation of Hydrocarbon $C_{19}H_{30}$ by Chromic Acid—A mixture of 1 mm of hydrocarbon $C_{19}H_{30}$, 7.5 gm. of chromium trioxide, and 6.0 ml. of sulfuric acid was heated under a reflux for 3 hours. Under these conditions methyl groups and their adjacent carbon atoms are converted to acetic acid (6). The reaction mixture was then subjected to steam distillation, the acid in the distillate determined by titration, and acetic acid isolated as the silver salt. The yield of acetic acid from the oxidation of different samples varied from 1.30 to 1.47 moles per mole of hydrocarbon $C_{19}H_{30}$. The isotope concentration of the carboxyl carbon of this acetic acid (degradation) was determined either by decarboxylation of silver acetate with bromine in carbon tetrachloride (11) or by pyrolysis of lithium acetate at 350–400° to yield acetone and lithium carbonate (12). Analysis of iodoform obtained from this acetone yielded the isotope concentration of the methyl group of acetic acid (degradation). In order to test the reliability of the procedures for the degradation of acetic acid, control

experiments were carried out with a sample of acetic acid-2-C¹⁴ of known specific activity. The results given in Table VI show that the degradation products have the expected isotope concentrations, except for iodoform which had a specific activity about 10 per cent lower than that calculated. No corrections have been made for this dilution in the values reported in Tables III and IV.

Dehydrogenation of Cholesterol by Palladium-Charcoal—0.40 gm. of isotopic cholesterol was intimately mixed with 0.15 gm. of 10 per cent palladium-charcoal and heated, after initial evacuation of the system, to 330-350° for 24 hours (8, 9). A gas trap cooled by liquid nitrogen was connected to the vacuum line in order to condense any hydrocarbons

Table VI
Degradation of Acetic Acid-2-C14

	Found	Calculated
	c.p.m.	c.p.m.
C14H2COOH	2040	
CH ₃ COCH ₃	2680	2720
CHI	3740	4080
CO ₂ *	20	0
CO ₂ †	0	0

^{*} By thermolysis of lithium acetate.

formed. After gas evolution had ceased, nitrogen was admitted to the system until atmospheric pressure had been reached. Nitrogen gas was passed slowly through the gas trap which was kept at -180° , and the exit gases were swept directly into a micro combustion furnace. Oxygen was fed into the combustion tube from a side arm. The combustion gases were passed through a Ba(OH)₂ solution in order to precipitate the carbon dioxide as BaCO₃. The baryta traps were changed every 20 minutes and the C¹⁴ content of the BaCO₃ samples measured separately (Samples 1 to 4, Table V). Since methane is the only hydrocarbon which has a sufficiently high vapor pressure at the temperature of liquid nitrogen, it is assumed that the gas removed is mainly methane. However, the varying isotope concentration of the different samples indicates that at least in Experiment I, Table V, the gas contained a second component.

Degradation of Cholesterol to Allocholanic Acid and Acetone—Cholesterol was converted to cholestane by way of dihydrocholesterol and cholestanone (13). The hydrazone of cholestanone was reduced to cholestane by the Wolff-Kishner method as described by Dutcher and Wintersteiner (14). The over-all yield from cholesterol was 52 to 57 per cent.

[†] By decarboxylation of silver acetate with bromine.

Oxidation of cholestane by chromic acid according to Windaus and Neukirchen (5) vields allocholanic acid and acetone. For the degradation of isotopic cholestane, 3 gm. of the steroid were suspended in 125 ml. of glacial acetic acid and 6.5 gm. of CrO₃ dissolved in 25 ml. of 80 per cent acetic acid were added in the course of 30 minutes. The reaction mixture was heated on the steam bath for 6 hours. Acetone was removed from the oxidation mixture by a slow stream of nitrogen and precipitated as the mercury complex (15) by passage through a boiling solution of mercuric sulfate. After completion of the oxidation, water was added to the flask and the reaction mixture was distilled to obtain the remaining acetone. The yield of acetone, calculated from the weight of the mercuryacetone complex, ranged from 10 to 20 per cent. Allocholanic acid was isolated according to the directions of Windaus and Neukirchen (5). The vield of crude potassium salt was about 10 per cent of the cholestane used. Purification of the allocholanic acid by recrystallization so diminished the vield as to prevent further use of this material for degradation studies. Attempts to increase the recovery by use of the procedure described by Fieser (16) for the oxidation of isoalkylnaphthoguinones were unsuccessful.

The C¹⁴ content of the acetone was determined by combustion of the mercury-acetone complex. For isotope analysis of the methyl groups of acetone, the mercury-acetone complex was decomposed by hydrochloric acid and the acetone converted to iodoform after distillation. The isotope content of the carbonyl group of acetone was calculated by difference.

Isotope Analyses—For C¹⁴ analysis all the samples were burned in a micro combustion apparatus, except the samples of mercury-acetone and iodoform, which were converted to carbon dioxide by the wet combustion procedure of Van Slyke and Folch (17). CO₂ was precipitated as barium carbonate and counted in a flow gas counter. Samples were counted for a sufficient length of time to insure less than 5 per cent probable error. The C¹⁴ values are given as counts of C¹⁴ per minute of BaCO₃ samples, corrected for infinite thickness. C¹⁸ was determined by mass spectrometric analysis.

SUMMARY

- 1. The biological synthesis of cholesterol has been investigated with the aid of acetic acid-1-C¹⁴, acetic acid-2-C¹⁴, and with acetic acid labeled by both C¹³ and C¹⁴.
- 2. The utilization of the 2 carbon atoms of acetic acid as a carbon source for the total molecule and for the cholesterol nucleus and side chain has been determined.
 - 3. Methyl groups of acetic acid have been identified as sources of carbon

atoms 18, 19, 26, 27, and presumably 17 in cholesterol. Carbon atom 25 and proabably carbon atom 10 are furnished by carboxyl groups of acetic acid.

BIBLIOGRAPHY

- 1. Bloch, K., and Rittenberg, D., J. Biol. Chem., 145, 625 (1942).
- 2. Bloch, K., and Rittenberg, D., J. Biol. Chem., 159, 45 (1945).
- 3. Bloch, K., Borek, E., and Rittenberg, D., J. Biol. Chem., 162, 441 (1946).
- 4. Rittenberg, D., and Bloch, K., J. Biol. Chem., 160, 417 (1945).
- 5. Windaus, A., and Neukirchen, K., Ber. chem. Ges., 52, 1915 (1919).
- 6. Kuhn, R., and L'Orsa, F., Z. angew. Chem., 44, 847 (1931).
- 7. Bergmann, E., and Bergmann, F., J. Chem. Soc., 1019 (1939).
- 8. Diels, O., Gaedke, W., and Koerding, P., Ann. Chem., 459, 1 (1927).
- 9. Ruzicka, L., Furter, M., and Thomann, G., Helv. chim. acta, 16, 812 (1933).
- 10. Weinhouse, S., Medes, G., and Floyd, N. F., J. Biol. Chem., 158, 411 (1945).
- 11. Hunsdiecker, H., and Hunsdiecker, C., Ber. chem. Ges., 75, 291 (1942).
- 12. Kroenig, W., Z. angew. Chem., 37, 667 (1924).
- 13. Bruce, W. F., Org. Syntheses, Coll. 2, 139 (1943).
- 14. Dutcher, J. D., and Wintersteiner, O., J. Am. Chem. Soc., 61, 1992 (1939).
- 15. Van Slyke, D. D., J. Biol. Chem., 83, 415 (1929).
- 16. Fieser, L. F., J. Am. Chem. Soc., 70, 3237 (1948).
- 17. Van Slyke, D. D., and Folch, J., J. Biol. Chem., 136, 509 (1940).

THE COLORIMETRIC ESTIMATION OF URINARY NEUTRAL STEROID ALCOHOLS*

BY LEWIS L. ENGEL, HELEN R. PATTERSON, HILDEGARD WILSON,†
AND MARION SCHINKEL

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, August 1, 1949)

The neutral non-ketonic alcohol fraction of urinary lipide extracts constitutes a substantial portion of the total neutral fraction as now separated by conventional procedures. A knowledge of the variations in the excretion of the compounds in this fraction would supplement the information obtained by other analytical procedures such as the Zimmermann reaction. Few methods have been proposed for the quantitative estimation of these alcohols. Electrometric titration of half succinates or half phthalates has been utilized by Lieberman et al. (1, 2) and the less specific antimony trichloride reaction has been used by Pincus (3). It was believed that a colorimetric method specific for carbinols would be useful both for isolation studies and for clinical purposes.

The wide and successful use of the Zimmermann reaction for the estimation of steroid ketones suggested that it might serve as a model. The reagent chosen was 3,5-dinitrophthalic anhydride. This compound contains the potentially chromogenic m-dinitrophenyl group and is capable of reacting with primary and secondary alcohols to yield alkali-soluble half esters. These half esters developed a red color upon treatment with methanolic potassium hydroxide in the absence of a ketone. The chromogenic properties of m-dinitrophenyl compounds in alkaline solution have been known for many years (4, 5) but in most instances the colors developed have been too transient to be suitable for quantitative measurements. It was found possible to define conditions for the application of this reaction to the quantitative estimation of urinary neutral steroid alcohols.

Method

Urinary Extracts—Duplicate aliquots of the neutral fraction of a 24 hour specimen in absolute ethanol, each usually amounting to one-tenth of the

* This work was supported by a grant from the National Cancer Institute, United States Public Health Service, administered by Dr. J. H. Means. A preliminary report was presented at the meeting of the American Society of Biological Chemists, Atlantic City, 1948 (Federation Proc., 7, 153 (1948)).

This is Publication No. 688 of the Cancer Commission of Harvard University. † Present address, Sloan-Kettering Institute for Cancer Research, New York.

total, are transferred to 10 ml. round bottom flasks and evaporated to dryness on the water bath with the aid of gentle suction. 1 ml. of toluene is added to each residue and removed by heating under gentle suction. After two more such treatments with toluene the residues are dried in vacuo over calcium chloride overnight. The size of the sample is adjusted if unusually high or low values are expected. Acetone solutions of pure steroid alcohols are simply evaporated to dryness in 10 ml. round bottom flasks immediately prior to esterification.

To each flask 24 mg. (100 μ eq.) of dinitrophthalic anhydride and 0.2 ml. of pyridine are added. The flasks are stoppered loosely and heated on the boiling water bath for 15 seconds. After cooling, the reaction mixture is diluted with 10 ml. of 0.1 N hydrochloric acid and transferred to a 60 ml. separatory funnel with 10 ml. of ether. The mixture is extracted with four 5 ml. portions of ether. The combined ether extracts are then washed with three 5 ml. portions of 0.1 N hydrochloric acid and five 5 ml. portions of water. The ether solution is then transferred to a 50 ml. volumetric flask with 10 ml. of ether and made up to volume with absolute methanol.

3 ml. aliquots of the methanol-ether solutions of the ester are transferred to colorimeter tubes and the solvents evaporated by immersing the tubes in a boiling water bath. The last traces of solvents are removed by the application of gentle suction. The residue is then dissolved in 7.0 ml. of methanol and treated with 3.0 ml. of 5.00 n potassium hydroxide¹ in methanol. A standard solution containing 0.5 μ eq. of cholesteryl hemi-3,5-dinitrophthalate is run with each set of unknowns. The readings are made at 510 m μ with a Coleman junior spectrophotometer 5 minutes after the addition of potassium hydroxide. The size of the aliquot taken for analysis should be adjusted so that a reading of 50 \pm 10 per cent transmission is obtained.

Color Reaction

Cholesteryl hemidinitrophthalate was prepared by the action of dinitrophthalic anhydride upon cholesterol in the presence of pyridine, and this ester was utilized for the study of the color reaction. Treatment of the ester with methanolic potassium hydroxide results in the immediate development of a pink color in the absence of a ketone.

The characteristics of the color reaction were then studied in detail in a Coleman junior spectrophotometer. The spectral absorption and extinc-

¹ The methanolic potassium hydroxide is prepared in 4 liter batches and standardized against 10 N HCl. The titer of this solution remains constant for 2 to 3 months when the bottle is protected by a soda-lime tube.

tion coefficients² from 400 to 600 m μ of cholesteryl hemidinitrophthalate and dinitrophthalic acid are shown in Fig. 1. It may be seen from the curves that steroid hemidinitrophthalates, of which group the cholesteryl

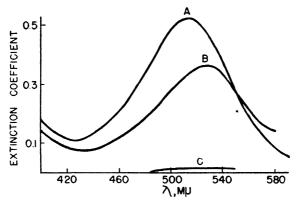


Fig. 1. Absorption spectra of cholesteryl hemi-3,5-dinitrophthalate in 1.52 N KOH in methanol (Curve A), 3,5-dinitrophthalic acid in 3.03 N KOH in methanol (Curve B), and 3,5-dinitrophthalic acid in 1.48 N KOH in methanol (Curve C).

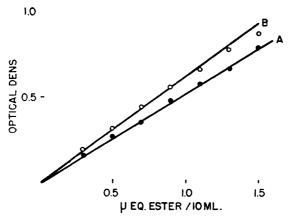


Fig. 2. Optical density-concentration relationships. Curve A, cholesteryl hemi-3,5-dinitrophthalate in 1.5 n KOH in methanol; Curve B, 2-methyl 3,5-dinitrophthalate in 1.5 n KOH in methanol.

ester is typical, show maximal absorption at 510 m μ , while 3,5-dinitrophthalic acid has its maximum at 530 m μ and requires an alkali concentration higher than that of the esters to develop comparable extinction.

² The extinction coefficient as used in this report is defined as the optical density of a solution containing 1 μ eq. of ester in 10 ml. of solution contained in a 7 \times $\frac{7}{4}$ intest-tube.

Beer's law is obeyed for all the compounds studied thus far, in the range 0.2 to $1.5 \,\mu\text{eq}$. of ester, although in the case of 2-methyl 3,5-dinitrophthalate some deviation occurs above $1.0 \,\mu\text{eq}$. (Fig. 2). The effect of alteration of the potassium hydroxide concentration on the extinction coefficient of

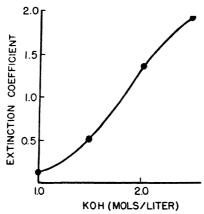


Fig. 3. Relation between extinction coefficient of cholesteryl hemi-3,5-dinitro-phthalate and potassium hydroxide concentration.

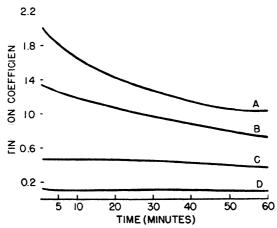


Fig. 4. The effect of potassium hydroxide concentration on the stability of the color developed by cholesteryl hemi-3,5-dinitrophthalate. Curve A, 2.49 N KOH; Curve B, 1.98 N KOH; Curve C, 1.47 N KOH; and Curve D, 1.02 N KOH.

cholesteryl hemidinitrophthalate is shown in Fig. 3. Although higher extinction coefficients may be obtained by increasing the potassium hydroxide concentration in the reaction mixture, the rate of fading of the color also increases, as can be seen from Fig. 4. The region of 1.5 N potassium hy-

droxide appears to represent a satisfactory compromise between sensitivity and color stability. At this concentration, the color has been found to be sufficiently stable so that readings may be made within 5 minutes after mixing the reagents.

Table I

Esterification of Cholesterol with \$,5-Dinitrophthalic Anhydride
10 µeq. of cholesterol were used in each experiment.

Variation	Anhydride	Pyridine	Heating time	Standing time	Esterifi- cation
APPRIANCE MAN AREA	µвq.	ml.	sec.	hrs.	per cent
Reactants	100	0.2	15	48	94
	100	0.5	15	48	84
	100	1.0	15	48	84
	100	1.5	15	48	69
Reactants	20	0.2	15	0	71
	40	0.2	15	0	84
	50	0.2	15	0	88
	70	0.2	15	0	81
	80	0.2	15	0	97
	150	0.2	15	0	85
Heating time	100	0.2	5	0	84
	100	0.2	30	0	88
			min.		
	100	0.2	1	0	90
	100	0.2	5	0	86
			sec.		
Standing time	100	0.2	15	0	90
.,	100	0.2	15	1	87
	100	0.2	15	2	85
	100	0.2	15	4	84
	100	0.2	15	16	89
	100	0.2	15	24	85

Esterification of Pure Steroid Alcohols

In order to adapt the color reaction to analytical purposes, it was necessary to define conditions under which maximal esterification would proceed, the variables being time, temperature, and the proportions of the reactants. Experiments were performed to determine the extent of esterification of cholesterol at varying time intervals. When a 10-fold excess of 3,5-dinitrophthalic anhydride (100 μ m) in 0.2 ml. of pyridine was used to esterify cholesterol, the reaction proceeded to its maximal extent almost instantly

at room temperature, as indicated in Table I. Increasing the volume of pyridine reduced the yield of ester. By using 0.2 ml. of pyridine and the 15 second heating period to insure complete solution of the reactants, it was found that a 5-fold excess of anhydride over alcohol led to maximal yields of ester. Nevertheless a 10-fold excess of anhydride is usually used in esterifying both pure compounds and urinary extracts.

The processing of the reaction mixture and the preparation of the ester for colorimetric analysis were found to be important, since substantial errors may be introduced by failure to effect complete removal of dinitrophthalic acid. It was found that, if the reaction mixture is diluted with water containing sufficient hydrochloric acid to half neutralize the pyridine, the esters may be extracted quantitatively with ether. The ether extracts

Table II

Ester.fication of Steroid Alcohols with 3,5-Dinitrophthalic Anhydride

Compound	Esterification* and standard deviation
	per cent
Cholesterol	93 ± 3
Epicholestanol	69 ± 4
Androsterone .	51 ± 5
Dehydroepiandrosterone	89 ± 3
Epiandrosterone	72 ± 5
Δ^{5} -Androstenediol-3 β , 17 β	$64 \pm 5\dagger$
Androstanediol- 3β , 17β	68 ± 5†
Pregnanediol- 3α , 20α	77 ± 4†
17-Methyltestosterone	3

^{*} Cholesterol hemidinitrophthalate was used as the standard.

may then be freed from pyridine by washing with dilute acid, and residual dinitrophthalic acid removed by washing with water. Control experiments in which the steroid alcohol was omitted gave extracts which yielded no color with 1.5 N methanolic potassium hydroxide even when 400 μ M of dinitrophthalic anhydride were used. Crystalline cholesteryl hemi-3,5-dinitrophthalate treated in a similar manner was recovered quantitatively.

The extent of esterification of a series of representative steroid carbinols was then studied, the conditions found optimal for the esterification of cholesterol being utilized. The yield of ester was based upon the extinction coefficient of pure cholesteryl hemi-3,5-dinitrophthalate which has been adopted as the reference standard. It will be seen from the data presented in Table II that in no case did the yield of ester reach the high values given by cholesterol. These results may be due to the failure of more hindered hydroxyl groups to react with the anhydride or possibly to de-

[†] Based upon esterification of both hydroxyl groups.

struction of the carbinols as a result of oxidative action of the dinitroanhydride. It is somewhat surprising that such variations in conditions as heating the reaction mixture for 1 hour, using rigorously dried pyridine, increasing the ratio of anhydride to alcohol, and substituting purified lutidine and quinoline for pyridine did not increase the extent of esterification of epicholestanol,³ the behavior of which is typical of the unreactive carbinols. The one tertiary alcohol tested, 17-methyltestosterone, failed to react significantly with dinitrophthalic anhydride.

It is interesting that all the pure esters of 3,5-dinitrophthalic acid (Table III) which have been prepared by the action of the anhydride upon the alcohol have their maximal absorption at the same wave-length (510 m μ). The size of the alkyl substituent which has been varied from C_1 to C_{27} appears to have little effect upon the extinction at this wave-length. It is

Table III $Crystalline\ Hemidinitrophthalates$ The absorption maximum is 510 m μ .

Alcohol	Extinction coefficient	кон
		moles per l
Cholesterol	0.52	1.52
Epicholestanol	0.53	1.51
Dehydroepiandrosterone		1.52
Androsterone	0.49	1.50
Methyl*	0.62	1.51

^{*} The structure of this ester is discussed in the text.

also noteworthy that the presence of a carbonyl group does not affect the wave-length of maximal absorption or the value of the extinction coefficient

Esterification of Urinary Extracts

In order to determine the applicability of this procedure to the estimation of alcohols in urinary extracts, aliquots of a pooled neutral fraction were esterified with dinitrophthalic anhydride and the color developed in the usual manner. The spectral characteristics of the ester mixture were similar to those of the pure steroid esters, although greater absorption in the region 400 to 440 m μ was observed. It was therefore necessary to make a correction for the intrinsic color of the extracts. Adherence to Beer's law was satisfactory both for ester mixtures of pooled neutral fractions and for neutral fractions from individual subjects chosen at random. The

³ The nomenclature and stereochemical configuration of the steroid compounds are based upon the recommendations of Fieser and Fieser (6).

esters obtained from urinary neutral fractions differ from those of the pure compounds in that the rate of fading of the color of the former was somewhat accelerated.

The range of steroid alcohol concentration over which the standard quantities of reagents would give reproducible results was examined and the results plotted in Fig. 5. It will be seen that maximal esterification was obtained when the ratio of anhydride to maximal alcohol determined was greater than 2.5. This contrasts with the behavior of cholesterol which required a 5-fold excess of anhydride for maximal esterification.

Since the neutral urinary extracts prepared in this laboratory are frequently stored in ethanol solution, it became necessary to devise a method for the complete removal of this solvent from the extract prior to esterifica-

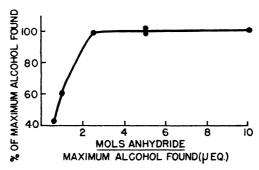


Fig. 5. The effect of varying the ratio of 3,5-dinitrophthalic anhydride to maximum alcohol determined in a urinary neutral fraction.

tion. A urinary extract was prepared without the use of ethanol and stored in toluene. It was found that when aliquots of this extract were evaporated to dryness three times with toluene under diminished pressure and stored overnight in a vacuum desiccator over calcium chloride, identical values for the steroid alcohol content were obtained with the extract alone and the extract after the addition of ethanol.

Application of Method

In order to determine whether this method would show changes in total urinary steroid alcohol excretion, 200 mg. of progesterone were administered to a male subject who had no obvious endocrinopathies. The daily excretion of ketosteroids (7) and steroid alcohols was followed for 7 days prior to the administration of progesterone. Progesterone was given intramuscularly in oil solution on 2 successive days. During these 2 days and the 2 following days, a marked increase in the excretion of steroid alcohols occurred (Fig. 6). In the last 3 days of the experiment the alcohol excretion dropped significantly below the pretreatment control level. Based

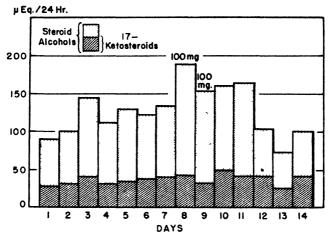


Fig. 6. The excretion of steroid alcohols and ketosteroids (microequivalents per day) by a normal male subject following the administration of two 100 mg. doses of progesterone.

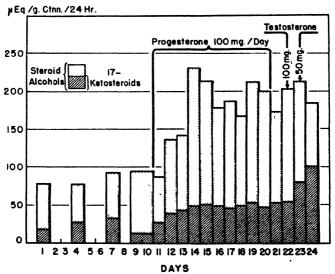


Fig. 7. The excretion of steroid alcohols and ketosteroids following the administration of progesterone and testosterone. The subject was a female with cancer of the breast. The excretion is expressed as microequivalents of ketosteroid and steroid alcohol per gm. of urinary creatinine per day.

upon the average pretreatment control level, the increased excretion corresponded to a recovery of 15 per cent of the administered progesterone calculated as pregnanediol.

A similar increase in the total urinary alcohol excretion was observed in a postmenopausal cancer patient treated with 100 mg. of progesterone daily for 9 days (Fig. 7) and testosterone for 2 days. In this instance there was a marked increase in the excretion of ketosteroids during the period of progesterone treatment. The significance of this change is not clear and is being studied. During the period of testosterone administration, the expected rise in ketosteroid excretion was observed, but on the final day of the experiment the non-ketonic alcohols appeared to drop to the control level, although the observation was discontinued too soon to be sure of this. The non-ketonic alcohol level was computed as the difference between the total alcohol value (microequivalents of cholesterol) and the ketosteroid value (microequivalents of dehydroepiandrosterone), assum-

Table IV
Urinary Excretion of Steroid Alcohols

Subjects*	Cholesterol
	μεq. per 24 hrs.
Men, normal	118
Women, normal (follicular phase)	115
pregnant	→ 454
Addison's disease, man (1 subject)†	50
" women (2 subjects)†	23
Panhypopituitarism, man	39
women	22

^{*} Unless otherwise indicated, the subjects were either laboratory personnel or patients under study by the staff of the Huntington Laboratory.

ing that equal numbers of carbonyl and alcohol groups are present in the ketosteroids.

This estimate is subject to error from two major sources, (1) the presence of non-alcoholic ketones in urine (artifacts of dehydration, etc.) which would tend to lower the apparent non-ketonic alcohol level and (2) the presence of non-steroidal alcohols and possibly amines which would give erroneously high values for the total alcohols. It is possible that a more accurate estimate of non-ketonic alcohol excretion would be obtained if the esterification with dinitrophthalic anhydride were carried out upon the non-ketonic fraction separated with Girard's reagent.

In Table IV are presented preliminary data on the excretion of steroid alcohols under various circumstances which indicate that the major source of the steroid alcohols is the adrenal cortex, with some contribution from the gonads. Further work on this problem is in progress and is directed toward an investigation of the excretion of steroid alcohols in normal sub-

[†] We are indebted to Dr. George W. Thorn and Dr. Peter Forsham for the specimens from these patients.

jects, patients with adrenal and gonadal malfunction, and cancer patients, particularly those treated with hormonal agents.

Structure of Esters

Theoretical considerations suggest that the steroid dinitrophthalates and the methyl dinitrophthalate prepared by a similar method are esters of the 2-carboxyl group. This view is supported by the properties of the isomeric 1-methyl ester prepared by the action of methanolic hydrogen chloride on dinitrophthalic acid. The behavior of these compounds and the dimethyl ester is summarized in Table V. In 1.5 N potassium hydroxide in methanol the 1-methyl ester color fades rapidly, but if the alkali concentration is doubled, the ester is converted quantitatively to dinitrophthalic acid within 15 minutes as measured by absorption at 530 m μ . In the cases of the 2-methyl and cholesteryl esters, in which the ortho effect is more pronounced, the fading is slower. If the ester solutions in 1.5 N potassium hydroxide are allowed to stand overnight, the colors are completely bleached. How-

TABLE V

Methyl 3,5-Dinitrophthalates

Ester	Preparation	Absorption maximum or color	
		1.5 N KOH in methanol	3.0 N KOH in methanol
1-Methyl 2-Methyl Dimethyl	Acid and methanol Anhydride and methanol Acid and diazomethane	Orange → colorless 510 mµ Orange → 510 mµ	Orange → 530 mµ 510 mµ

ever, if the concentration of potassium hydroxide is then increased to 3 N, color corresponding to 65 per cent of the theoretical amount of dinitrophthalic acid (absorption maximum 530 m μ) is developed. The dimethyl ester which was prepared by the action of diazomethane upon dinitrophthalic acid gives an immediate deep orange-red color in 1.5 N potassium hydroxide in methanol. After a few moments the orange changes to the typical pink of the 2-methyl ester and the solution shows maximal absorption at 510 m μ .

EXPERIMENTAL

All melting points were measured on a Fisher-Johns instrument and were corrected unless otherwise stated.

Elementary analyses marked F. were semimicroanalyses performed by Dr. Carol K. Fitz, Melrose, Massachusetts. Those marked E. were microanalyses by the Elek Microanalytical Laboratories, Los Angeles, California.

The Coleman junior spectrophotometer was used for all spectrophotometric measurements.

Dinitrotetralin—Commercial grade tetralin was nitrated with fuming nitric acid and concentrated sulfuric acid by a modification of the method of Schroeter (8). The eutectic mixture of 1,2- and 1,3-dinitrotetralin, m.p. 71-74°, was obtained in 39 to 44 per cent yield. Material having this melting point was sufficiently pure for further treatment. This material has also been prepared by the nitration of tetralin with fuming nitric acid alone (9).

- 3,5-Dinitrophthalic Acid—Oxidation of the eutectic mixture of the dinitrotetralins by repeated treatments on the steam bath with 30 per cent nitric acid was carried out essentially as described by Schroeter (8). After recovery from the barium salt, the crude acid was crystallized from etherligroin (b.p. 60-90°) and then melted at 229-230° with decomposition in a sealed tube. By reoxidation of the acid mother liquors a yield of 50 per cent was obtained. Acid melting above 225° was sufficiently pure for conversion to the anhydride. The acid has also been prepared by the oxidation of 2-methyl-3,5-dinitrobenzoic acid with nitric acid in a sealed tube (10) and by the nitric acid oxidation of 2,4,5,7-tetranitronaphthol (11).
- 3,5-Dinitrophthalic Anhydride—The acid was dehydrated by refluxing with freshly distilled acetic anhydride as described by Eder and Widmer (10) and was obtained in about 75 per cent yield. The pure anhydride melted at 162–164° and had a neutral equivalent of 119. Only the first fraction was collected and the mother liquors were hydrolyzed with water and subjected to reoxidation.

1-Methyl 3,5-Dinitrophthalate—A solution of 4 gm. of 3,5-dinitrophthalic acid in 400 ml. of 1 per cent hydrogen chloride in methanol was boiled under a reflux for 12 hours. The solvent was removed under reduced pressure and the residue dissolved in a small volume of methanol and distilled to dryness. The oily residue was finally dried in vacuo over potassium hydroxide.

The separation of the ester from unchanged acid by means of counter-current distribution in isopropyl ether-water was unsuccessful. The recovered mixture was taken up in ether and chromatographed on a column of magnesium silicate No. 16 (1 part)⁴ and Celite No. 545 (3 parts) measuring 34×125 mm. Elution was carried out with benzene, benzene-ether mixtures, and ether. The material eluted by benzene was recrystallized five times from benzene-ether and once from chloroform. The pure 1-methyl ester obtained melted at $177-179^{\circ}$.

C₉H₅N₂O₈. Calculated, N 10.4, neutral equivalent 270 Found, "10.3 (F.), neutral equivalent 267

2-Methyl 3,5-Dinitrophthalate—3,5-Dinitrophthalic anhydride was dissolved in absolute methanol and, after standing at room temperature for

⁴ Philadelphia Quartz Company, Berkeley, California.

1½ hours, the solution was evaporated under reduced pressure. The residue was crystallized twice from ether-pentane mixture and yielded pure ester which melted at 178–179° and depressed the melting point of 1-methyl dinitrophthalate.

Dimethyl 3,5-Dinitrophthalate—The reaction of 3,5-dinitrophthalic acid with excess diazomethane yielded the dimethyl ester which melted at 126–126.5° after four crystallizations from ether.

Cholesteryl Hemi-3,5-dinitrophthalate—A mixture of 1.93 gm. (5 mm) of cholesterol and 11.90 gm. (50 mm) of dinitrophthalic anhydride was dissolved in 25 ml. of pyridine by heating for about 30 seconds on the water bath. After cooling, the reaction mixture was diluted with 475 ml. of water and 25 ml. of concentrated hydrochloric acid and extracted with 300 ml. and four 100 ml. portions of ether. The ether extracts were washed with nine 100 ml. portions of 0.1 n hydrochloric acid and five 100 ml. portions of water. After adding 25 ml. of benzene, the ether solution was evaporated to dryness under reduced pressure. The residue was recrystallized five times from aqueous acetone to yield 2.49 gm. of cholesteryl hemidinitrophthalate, m.p. 215–216°, with softening and darkening at 206°.

Epicholestanyl Hemi-3,5-dinitrophthalate—A mixture of 390 mg. (1 mm) of epicholestanol and 2.38 gm. (10 mm) of 3,5-dinitrophthalic anhydride was dissolved in 10 ml. of pyridine by heating on the boiling water bath for 15 seconds. After cooling, the flask was stoppered and allowed to stand at room temperature for 24 hours. The reaction mixture was then diluted with 145 ml. of water and 5 ml. of concentrated hydrochloric acid and extracted with three 50 ml. portions and one 30 ml. portion of ether. The ether solution was washed with three 30 ml. portions of 0.1 N hydrochloric acid and five 30 ml. portions of water, then diluted with 25 ml. of benzene. After evaporation to dryness, the residue was recrystallized four times from aqueous acetone and three times from aqueous methanol: Epicholestanyl hemi-3,5-dinitrophthalate melts at 211–212.5° with softening at 205°.

Dehydroepiandrosterone Hemi-3,5-dinitrophthalate—A mixture of 144 mg. of dehydroepiandrosterone and 1.9 gm. of 3,5-dinitrophthalic anhydride was dissolved in 7 ml. of pyridine and allowed to stand at room temperature for 22 hours. The reaction mixture was then diluted with 70 ml. of 2.5 N hydrochloric acid and extracted with 40 ml. and then with four 25

ml. portions of ether. The combined ether extracts were washed with four 25 ml. portions of 0.1 n hydrochloric acid, then with three 25 ml. portions of water, dried over sodium sulfate, and evaporated to dryness.

The product was further purified by an eight-transfer counter-current distribution in separatory funnels with 20 per cent ethyl acetate in hexane as the upper phase and 50 per cent ethanol as the lower phase. The volume of each phase was 40 ml. At the end of the distribution 15 ml. of absolute ethanol were added to each funnel to make the system homogeneous. After measurement of the volume, aliquots were removed from each funnel and analyzed for ester. The contents of Funnels 2, 3, and 4 which contained the bulk of the ester were combined and evaporated to dryness under reduced pressure. The crystalline residue was recrystallized twice from aqueous ethanol and the product melted at 222–224° with decomposition.

C₂₇H₂₀N₂O₃. Calculated, N 5.32; found, N 5.43 (E.)

Preparation of Androsterone Hemi-3,5-dinitrophthalate and Recovery of Androsterone from Reaction Mixture—A mixture of 100 mg. (0.34 mm) of androsterone and 785 mg. (3.4 mm) of dinitrophthalic anhydride was dissolved in 7 ml. of pyridine and heated on the water bath for 15 seconds. After cooling, the reaction mixture was diluted with 100 ml. of water containing 3.5 ml. of concentrated hydrochloric acid and extracted with 60 ml. and four 35 ml. portions of ether. The combined ether extracts were washed three times with 0.1 N hydrochloric acid and five times with water, dried over sodium sulfate, and taken down to dryness. Analysis showed that 43 per cent of the androsterone had been esterified. residue was extracted with six 10 ml. portions of boiling hexane and filtered. The hexane solution was evaporated to a small volume and the crystalline precipitate which formed was filtered off and dried. 4.5 mg. of material, m.p. 184°, were obtained. No depression of the melting point was observed upon mixture of this material with an authentic specimen of androsterone.

The hexane-insoluble material was recrystallized from methanol and yielded 36 mg. of androsterone hemidinitrophthalate, m.p. 227-228°, with decomposition.

C27H22N2O3. Calculated, N 5.30; found, N 5.14 (E.)

SUMMARY

1. The action of methanolic potassium hydroxide upon certain steroid hemidinitrophthalates gives colored products which are of sufficient stability to be useful for quantitative measurements.

- 2. Secondary alcohols of the steroid group may be converted to chromogenic esters in 51 to 93 per cent yield by the action of 3,5-dinitrophthalic anhydride in pyridine.
- 3. This reaction has been applied to the estimation of urinary steroid alcohols and has yielded information concerning the origin and amounts of these compounds present in urinary extracts.
- 4. The anticipated increase in urinary steroid alcohol excretion was observed following the administration of progesterone to a normal male subject and to a female cancer patient.

We are indebted to the Schering Corporation for the samples of androstanediol and androstenediol.

We wish also to express our thanks to Miss Gladys Ekman for the ketosteroid determinations reported.

BIBLIOGRAPHY

- Lieberman, S., Fieser, L. F., Dobriner, K., and Rhoads, C. P., Endocrinology, 35, 223 (1944).
- 2. Dobriner, K., Lieberman, S., and Rhoads, C. P., J. Biol. Chem., 172, 241 (1948).
- 3. Pincus, G., Endocrinology, 35, 227 (1944).
- 4. Meyer, V., and Stadtler, O., Ber. chem. Ges., 17, 2778 (1884).
- 5. von Bittó, B., Ann. Chem., 269, 377 (1892).
- Fieser, L. F., and Fieser, M., Natural products related to phenanthrene, American Chemical Society monograph series, New York, 3rd edition (1949).
- 7. Nathanson, I. T., and Wilson, H., Endocrinology, 33, 189 (1943).
- 8. Schroeter, G., Ann. Chem., 426, 43 (1922).
- Makarov-Zemlyanskii, Y., and Bihishev, V. P., J. Gen. Chem. U. S. S. R., 7, 1280 (1937).
- 10. Eder, R., and Widmer, C., Helv. chim. acta, 6, 966 (1923).
- 11. Merz, V., and Weith, W., Ber. chem. Ges., 15, 2708 (1882).

LACTIC DEHYDROGENASE OF LIVER AND ITS RELATION TO THYROID ACTIVITY IN THE RAT*

By CARL S. VESTLING AND AUGUSTA A. KNOEPFELMACHER†

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

(Received for publication, August 8, 1949)

The relationship between the thyroid hormone and enzyme activity has been studied in this work. Changes in metabolic rate have been reported by various investigators to parallel certain changes in enzyme activity.

Thus enzyme studies on liver homogenates from thyrotoxic rats have shown an increase in cytochrome oxidase, succinoxidase (1), and p-amino acid oxidase activities (2). A decrease in the concentration of these enzymes and of cytochrome c (3) was noted following thyroidectomy. Since lactic acid metabolism seems to be disturbed in hyperthyroidism, as evidenced by increased blood lactic acid values and decreased lactate tolerance (4-7), the present work was undertaken to find out whether changes in liver lactic dehydrogenase activity could be observed similar to those reported for the above oxidative enzymes. Lactic acid oxidation has been studied for the most part in heart muscle systems (8-12), but data have also been reported for liver and other organs (10, 13).

In an approach to this problem, it was found that optimum conditions had not yet been described for a study of liver lactic dehydrogenase. In this report such conditions are presented in conjunction with a comparison of data obtained from normal and from (1) thyroparathyroidectomized, (2) thiouracil-treated, and (3) thyroid-fed rats.

EXPERIMENTAL

Male rats¹ which had been fasted for 16 to 24 hours were stunned by a blow on the head, decapitated, and exsanguinated. The liver was removed rapidly, placed on ice, and appropriate portions weighed directly into a homogenizing tube. 4 volumes of ice-cold redistilled water were added and the mixture homogenized for 4 minutes in a Potter-Elvehjem

- * From a thesis submitted by Augusta A. Knoepfelmacher in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois, August, 1948.
- † Present address, Department of Chemistry, State College of Washington, Pullman, Washington.
- ¹ The albino rats used were obtained from the University of Illinois inbred colony and from Sprague-Dawley, Inc., Madison, Wisconsin.

homogenizer (14). The resulting homogenate was filtered through cheese-cloth and centrifuged in a clinical centrifuge at 2500 R.P.M. for 4 minutes. The supernatant liquid was diluted with water to give an approximately 3 per cent extract and was used in all further work, except in early experiments when a comparison of whole versus centrifuged homogenates was made. All the enzyme activity was found to be in the supernatant liquid. The dry weights of all samples were determined by evaporation of the extract to constant weight.

Oxygen uptake was measured by means of the conventional Warburg apparatus at 38°. All the reactants except diphosphopyridine nucleotide (DPN) were added to the main compartment of the flask, which was kept at room temperature before being placed in the bath. DPN solutions were pipetted into the side arm of the Warburg flasks and added to the main compartment after a 5 minute equilibration period.

All acidic components of the system were adjusted to pH 8.0 with sodium hydroxide, except DPN which was adjusted to pH 7.0. The components of the final reaction mixture were as follows: 0.5 ml. of 3.5×10^{-4} m cytochrome c, 0.3 ml. of a 1 per cent solution of DPN (50 per cent purity), 0.4 ml. of 0.2 m sodium phosphate buffer or 0.8 ml. of 0.1 m sodium diethyl barbiturate (veronal) at pH 8.0, 0.8 ml. of 0.4 m pl-lactate, 0.2 to 0.4 ml. of a 3 per cent rat liver extract, and water to make a total volume of 3 ml. (0.2 ml. of 0.25 m nicotinamide was also added in a number of experiments.)

Cytochrome c was prepared from frozen horse heart according to the method of Keilin and Hartree (15) and standardized photometrically after momentary heating to 95° and filtering and discarding any precipitate formed. DPN was prepared from bakers' yeast according to the method of LePage (16). Nicotinamide was a Merck product, and the lactic acid used was either the Mallinckrodt (N. F. VIII, 85 per cent, sp. gr. 1.206) or Coleman and Bell (U. S. P., 85 per cent) product. The concentrated lactic acid was diluted with redistilled water, boiled to hydrolyze lactides and lactic anhydrides, and neutralized with sodium hydroxide. The sodium lactate solution must be made up from concentrated lactic acid at least every 4th day. On standing in the refrigerator, lactate solutions allow bacterial growth, which results in the conversion of lactic acid to pyruvic acid; the latter inhibits lactate oxidation (10). Meyerhof and Lohmann (17) have shown that in tissues L-lactic acid is oxidized at the same rate as DL-lactic acid; that is, the presence of the unnatural isomer does not inhibit the utilization of the natural isomer.

Pyruvate analyses were performed colorimetrically, according to the method outlined in Umbreit et. al. (18), on trichloroacetic acid filtrates of the incubation mixture. Lithium pyruvate was used in the preparation of the standard curve

Thyroid preparations were obtained from The Maltine Company. One sample of thyroglobulin contained 0.82 per cent total iodine and 0.18 per cent thyroxine iodine; another preparation of desiccated thyroid material contained 0.87 per cent total iodine and 0.20 per cent thyroxine iodine.

The data are expressed as $Q_{0_2}^{10}$ values. $Q_{0_3}^{10}$ values are calculated on the basis of microliters of oxygen consumed per 10 minutes per mg. of dry weight of tissue, and this value is multiplied by 6 to give the potential oxygen consumption per hour. Oxygen uptake was based on the first 10 minute period of incubation, except in a few experiments in which the uptake was greater in the second 10 minute period. After 20 minutes the oxygen consumption falls off slowly owing to the inhibitory action of the end-product, pyruvic acid.

All rats were 2 months old at the beginning of experiments. Hypothyroidism was produced by either thyroparathyroidectomy or by feeding the rats stock diets (Purina fox chow) containing 1 per cent thiouracil. Hyperthyroidism was elicited by feeding stock diets containing various amounts of thyroid-active material. The treated animals were fed ad libitum. In the case of thyroidectomized rats, two control groups of the same age were used: one group of rats was fed ad libitum; the other group was pair-fed with the thyroidectomized rats. The animals were used 4 months after removal of the thyroid gland. For thiouracil and thyroid-fed rats, normal males served as controls; they had free access to food. (At first pair-fed controls were used with the thiouracil group. But since it was shown later that rats which had been on a limited food intake for several months had the same lactic dehydrogenase activity as normal rats, pair-feeding of this group was discontinued.)

Results

The reversible oxidation of lactate to pyruvate in liver homogenates is assumed to proceed by interaction of lactate with DPN on the surface of the lactic dehydrogenase. Subsequent steps then involve cytochrome reductase, cytochrome c, cytochrome oxidase, and oxygen.

The oxidation of malate to oxalacetate proceeds through similar steps (13). The reoxidation of reduced DPN through the cytochrome system has been discussed by Potter in connection with the malic dehydrogenase system (13); he has shown that DPN and cytochrome c have to be added in excess to liver homogenate systems, while cytochrome reductase and cytochrome oxidase are already present in excess. If this is the case in the system described in the present report, then lactic dehydrogenase becomes the rate-limiting enzyme. This could be proved conclusively by adding graded amounts of pure liver lactic dehydrogenase to a system and observing the changes in oxygen uptake; however, the enzyme has

not yet been purified. But since it is reasonable to assume that the reoxidation of reduced DPN proceeds through the same steps whether lactic or malic acid is the hydrogen donor, the imposition of malate oxidation on lactate oxidation should show whether lactic dehydrogenase is the rate-determining enzyme. The addition of malate and glutamate to the lactic dehydrogenase system increased the oxygen uptake in several experiments by about 60 per cent. (Glutamate was added to remove the end-product of malate oxidation, oxalacetate, by transamination (13).)

TABLE I Optimum Conditions for Liver Lactic Dehydrogenase System

The data for each compound added were obtained from separate experiments. 0.4 ml. of 3 per cent liver extract was added per flask; the other components of the reaction mixture were added as described in the text.

DL-La	DL-Lactate		Cytochrome c		DPN		amide	Phosp	hate
Molarity	Q10 O2	Molarity × 10-5	Q _{O2} ¹⁰	Molarity × 10 ⁻⁴	Q _{O₂} ¹⁰	Molarity	Q10 03	Molarity	Q10 O3
0	2.0	0	2.0	0	4.0	0	83.9	0*	93.0
0.01	39.0	1.03	42.8	0.25	32.7	0.008	80.5	0.027	87.3
0.024	59.0	3.10	71.2	1.26	58.1	0.017	74.4		
0.049	68.5	4.12	73.3	2.52	64.3	0.034	80.9		
0.098	86.7	6.20	73.8	3.71	73.3				
0.194	81.9			8.0	73.8				
0.267	79.7			12.6	72.6				

* Veronal buffer was used (0.027 m). The Q_{02}^{10} values in the fourth and sixth columns were obtained in early experiments in which lactate solutions were used after more than a week's storage. This results in diminished oxygen uptake, as explained in the text.

The optimum concentrations of the components of the system have been determined, and the data are condensed in Table I. The need for added cytochrome c and DPN is clearly indicated. The quality of the DPN added is of considerable importance. DPN not purified by the Le-Page procedure (16) gave high and irregular blank values (oxygen uptake in the absence of lactate); in the presence of purified DPN consistently low blank values were obtained. Mann and Quastel (19) reported that DPN is rapidly destroyed in tissue homogenates and that nicotinamide inhibits this breakdown. Potter (13) made use of this fact by adding nicotinamide to the malic dehydrogenase system. Under the conditions used here, no decrease in oxygen uptake could be observed in the absence of added nicotinamide even after a 60 minute incubation period (Table I). As indicated in Table I, inorganic phosphate is not a necessary component of the system; veronal buffer is equally effective. Since Fig. 1 indicates

that the optimum pH is about 8.0, veronal, which is a better buffer at this pH than phosphate, is to be preferred in the system. Fig. 1 illustrates the necessity of careful pH control. If the determinations had been carried out at pH 7.0, for example, the slightest variations in final pH values would have resulted in changes in oxygen uptake not related to the state of the animals. At about pH 8.0, small changes in pH values do not result in appreciable changes in oxygen consumption.

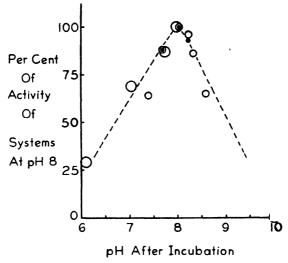


Fig. 1. Determination of the optimum pH for the liver lactic dehydrogenase system. The components of the reaction mixture are described in the text. Phosphate and veronal buffers were used. The data were obtained from four separate experiments. O, normal rats, veronal buffer; O, thiouracil-fed rat, phosphate buffer; •, hyperthyroid rat, phosphate buffer.

Fig. 2 shows a direct proportionality between the oxygen uptake of the system and the tissue concentration when 1 to 4 mg. of tissue (dry weight) is added per flask. The data indicate the suitability of this system for tissue assay.

In order to correlate oxygen consumption with lactate utilization, pyruvate analyses were performed at the end of a 20 minute incubation period. Added pyruvate is not oxidized in the system. The results demonstrate that for 1 mole of oxygen consumed slightly more than 2 moles of pyruvate are formed (2.2 to 2.5 moles). The high pyruvate values obtained can probably be explained. During the time (1 to 2 minutes) aliquots of the homogenate were transferred from the Warburg flask into trichloroacetic acid, oxygen consumption continued, but was not recorded.

Since pyruvate accumulates in the system and is known to inhibit

lactate oxidation (Table II), glutamate was added to effect the removal of pyruvate through transamination. This principle has been used successfully to remove oxalacetate in the assay for malic dehydrogenase (13). The addition of glutamate to the complete system increases the oxygen uptake (Table II) in an irregular fashion. The end-product of the proposed transamination reaction, α -ketoglutarate, is not utilized in the system. Das (20) reported that sodium fluoride inhibits heart lactic dehydrogenase activity. As Table II shows, the effect of sodium fluoride on liver lactic dehydrogenase activity was found to be negligible.

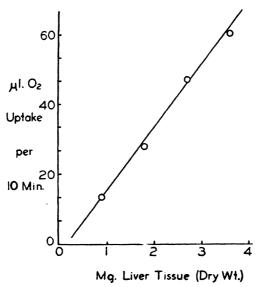


Fig. 2. Relationship between oxygen uptake and tissue concentration in the rat liver lactic dehydrogenase system. The components of the reaction mixture are described in the text, except that 0.1 to 0.4 ml. of 3 per cent liver extract was used.

Many conflicting experiments are reported in the literature on the addition of thyroglobulin and thiouracil *in vitro*. In Table II, it is shown that the addition of either thyroglobulin or thiouracil *in vitro* does not affect the oxygen uptake.

Fig. 3 shows the rate of lactate oxidation in the system. Oxygen uptake is linear with respect to time for the first 20 minute period and then decreases slowly. In the absence of the substrate, lactate, oxygen consumption is negligible. The addition of glutamate, α -ketoglutarate, or pyruvate in place of lactate does not increase the oxygen uptake of the system significantly above that of the blank. Pyruvate is not oxidized, even when magnesium ions and adenosine triphosphate are added to the components of the reaction mixture.

C. S. VESTLING AND A. A. KNOEPFELMACHER

Table II

Effect of Adding Certain Substances on Oxygen Uptake of Liver Lactic

Dehydrogenase System

The components of the reaction mixture are described in the text.

Experiment No.	Addition to complete system	Final concentration	$Q_{\mathbf{O_8}}^{10}$
		и	
73	None		82.7
	Sodium fluoride	0.013	77.1
103	None		82.1
	Sodium fluoride	0.013	84.4
	" pyruvate	0.001	68. 3
87*	None		57.8
	Sodium pyruvate	0.013	15.5
	" pyruvate	0.027	7.8
104	None		80.2
	Sodium L-glutamate	0.05	100.2
97†	None		98.2
	Sodium L-glutamate	0.067	137.4
79	None		79.2
	Thiouracil	0.001	83.4
82a	None		88.0
	Thyroglobulin	0.3%	86.4

^{*} Thyroid-fed rat.

[†] Thiouracil-fed rat.

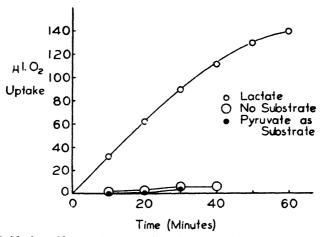


Fig. 3. Oxidation of lactate in the rat liver lactic dehydrogenase system. The components of the reaction mixture are described in the text. 1.8 mg. of liver (dry weight) were added per flask.

A satisfactory procedure for the assay of lactic dehydrogenase activity in liver homogenates enabled us to study the relation to thyroid activity.

Table III summarizes the results obtained with differently treated rats. There were no significant differences in enzyme activity among thyroidectomized, normal, and pair-fed control animals of the same age. Thiouracil treatment, however, resulted in a small, but statistically significant increase in enzyme activity.

Since it is doubtful whether the thyroid gland can be removed completely, the question may be raised whether the rats had been properly thyroidectomized. Although preliminary basal metabolic studies indicated that this was the case, changes in body and organ weights further demonstrated the striking decrease in growth following the removal of the thyroid gland. Thiouracil administration resulted in a similarly decreased growth

Table III

Comparison of Liver Lactic Dehydrogenase Activity in Normal and Treated Rats

Treau me nt	No. of	No. of Q 02			P	
Treatment	animals*	Mean ± S.D.	Range	it		
None (age, 2½ mos.) Thiouracil-fed Thyroid-fed None (age, 6 to 7 mos.) Thyroidectomy Pair-fed	4 (11) 7 (12) 3 (9) 4 (7)	$84.3 \pm 3.6 96.0 \pm 3.0 67.7 \pm 5.9 87.0 \pm 7.1 87.6 \pm 3.0 89.4 \pm 1.8$	57.8-73.2 84.7-96.6 82.8-91.0	5.4 6.4	<0.01 <0.01	

^{*} The figures in parentheses indicate the total number of animals used, including preliminary experiments which, when analyzed statistically, yielded similar comparative results.

rate, decreased food consumption, and decreased spleen weights; liver weights were the same as for normal rats. Thyroid treatment, on the other hand, was accompanied by an increased food consumption, a decrease in body weight in all but one case, and no noticeable change in organ size. There is no apparent correlation between the amount (0.2 to 1.0 per cent of the diet) and nature (thyroglobulin or desiccated thyroid) of the administered thyroid material and liver lactic dehydrogenase activity.

Liver homogenates of thyroid-fed rats showed a 22 per cent decrease in lactic dehydrogenase activity (Table III), which was found to be statistically significant. There is a difference of 42 per cent between thyroid-fed and thiouracil-fed rats.

DISCUSSION

The increased basal metabolic rate of the body accompanying hyperthyroidism seems to be reflected in an increased activity in enzyme systems

 $[\]dagger t$ values were calculated according to Chambers (21) and P values were obtained from the tables compiled by Fisher (22).

so far investigated. The results obtained here appear to offer a contrast. However, on the assumption that the assay of enzymes in tissue homogenates reflects what occurs in the intact organism, the changes reported here can be correlated with results obtained by entirely different experimental approaches to the thyroid problem.

A literature survey pertinent to the relationship between hyperthyroidism and lactic dehydrogenase activity reveals the following facts: (1) Experimental hyperthyroidism is characterized by a marked liver glycogen deficiency (23-29). (2) One source of liver glycogen is lactic acid (30-33), and the mechanism of glycogen formation is believed to consist of a reversal of the steps involved in the glycolytic process (34). (3) Lactate utilization is disturbed in hyperthyroidism, as evidenced by high resting and working blood lactic acid values and decreased lactate tolerance (4-7). (4) Lactate utilization is also disturbed in serious clinical liver damage; this is the basis of the lactate tolerance test for liver function (35-37). (5) Clinical hyperthyroidism is frequently accompanied by liver damage (38).

A'correlation of the results obtained in this paper with the observations cited above supports the hypothesis that the low liver glycogen, high blood lactic acid, and decreased lactate tolerance accompanying hyperthyroidism are at least to some extent due to the decreased capacity of the liver to synthesize glycogen from lactic acid.

It cannot be argued, however, that general liver damage is responsible for the decrease in enzyme activity, since succinoxidase, cytochrome oxidase (1), and p-amino acid oxidase (2) have been found to be increased in liver tissue of thyrotoxic animals. Furthermore, an increase in oxygen consumption of intact liver slices from thyroid-fed rats has been repeatedly demonstrated. It is possible that under the influence of excess thyroid hormone there is a general increase in non-specific oxidation in the liver at the expense of a specialized liver function; namely, glycogenesis from lactic acid.

SUMMARY

- 1. An assay for rat liver lactic dehydrogenase has been reported and the properties of the system studied.
- 2. A study has been made of the activity of rat liver lactic dehydrogenase at different levels of thyroid function. Thyroid administration resulted in a statistically significant decrease in enzyme activity. Thiouracil administration was accompanied by a small increase, and thyroidectomy did not affect the rate of lactate oxidation in the liver homogenates.
- 3. Experimental evidence has been reviewed in support of the hypothesis that hepatic glycogenesis from lactic acid may be disturbed in hyperthyroidism.

The authors wish to thank W. J. Frajola for the preparation of cytochrome c; Dr. A. V. Nalbandov, Department of Animal Science, University of Illinois, for a supply of thiouracil; Dr. G. E. Phillips of The Maltine Company for samples of desiccated thyroid and thyroglobulin; and Dr. F. J. Rudert of the Red Star Yeast and Products Company for making quantities of fresh bakers' yeast available to us.

BIBLIOGRAPHY

- 1. Tipton, S. R., and Nixon, W. L., Endocrinology, 39, 301 (1946).
- 2. Klein, J. R., J. Biol. Chem., 128, 659 (1939).
- 3. Drabkin, D. L., Federation Proc., 7, 152 (1948).
- 4. Dresel, K., Deut. med. Wochschr., 55, 259 (1929).
- 5. Zondek, H., Deut. med. Wochschr., 55, 345 (1929).
- 6. Bier, A., Klin. Wochschr., 8, 1306 (1929).
- 7. Eichler, O., and Sanders, W., Klin. Wochschr., 9, 1618 (1930).
- 8. Boyland, E., and Boyland, M. E., Biochem. J., 28, 1417 (1934).
- von Euler, H., Adler, E., Günther, G., and Hellström, H., Z. physiol. Chem., 245, 217 (1937).
- 10. Green, D. E., and Brosteaux, J., Biochem. J., 30, 1489 (1936).
- 11. Corran, H. S., Green, D. E., and Straub, F. B., Biochem. J., 33, 793 (1939).
- 12. Straub, F. B., Biochem. J., 34, 483 (1940).
- 13. Potter, V. R., J. Biol. Chem., 165, 311 (1946).
- 14. Potter, V. R., and Elvehjem, C. A., J. Biol. Chem., 114, 495 (1936).
- 15. Keilin, D., and Hartree, E. F., Biochem. J., 39, 289 (1945).
- 16. LePage, G. A., J. Biol. Chem., 168, 623 (1947).
- 17. Meyerhof, O., and Lohmann, K., Biochem. Z., 171, 421 (1926).
- 18. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1947).
- 19. Mann, P. J. G., and Quastel, J. H. Biochem. J., 35, 502 (1941).
- 20. Das, N. B., Biochem. J., 31, 1116 (1937).
- 21. Chambers, E. G., Statistical calculations for beginners, London (1943).
- Fisher, R. A., Statistical methods for research workers, London, 7th edition (1938).
- 23. Cramer, W., and Krause, R. A., Proc. Roy. Soc. London, Series B, 86, 550 (1913).
- 24. Kuriyama, S., J. Biol. Chem., 33, 193 (1918).
- 25. Boesl, O., Biochem. Z., 202, 299 (1928).
- 26. Lawrence, R. D., and McCance, R. A., Biochem. J., 25, 570 (1931).
- 27. Coggeshall, H. C., and Greene, J. A., Am. J. Physiol., 105, 103 (1933).
- 28. Liebig, H., Z. ges. exp. Med., 109, 715 (1941).
- 29. McIver, M. A., and Winter, E. A., J. Clin. Invest., 21, 191 (1942).
- 30. Cori, C. F., and Cori, G. T., J. Biol. Chem., 81, 389 (1929).
- 31. Grant, R., J. Physiol., 80, 41 (1933).
- 32. Hodgson, T. H., Biochem. J., 27, 1157 (1933).
- 33. Kerly, M., and Reid, C., J. Physiol., 84, 302 (1935).
- 34. Lardy, H. A., and Ziegler, J. A., J. Biol. Chem., 159, 343 (1945).
- 35. Schumacher, H., Klin. Wochschr., 7, 1733 (1928).
- 36. Snell, A. M., and Roth, G. M., J. Clin. Invest., 11, 957 (1932).
- 37. Soffer, L. J., Arch. Int. Med., 60, 882 (1937).
- 38. Lichtman, S. S., Ann. Int. Med., 14, 1199 (1941).

THE DETERMINATION OF GLUTAMIC ACID DEHYDROGENASE IN TISSUE HOMOGENATES*

By J. H. COPENHAVER, JR., W. H. McSHAN, AND ROLAND K. MEYER (From the Department of Zoology, University of Wisconsin, Madison)

(Received for publication, August 20, 1949)

In 1938, two groups of workers published extensive studies of glutamic dehydrogenase in animal tissues. The enzyme preparation of von Euler, Adler, Günther, and Das (1) was a dialyzed extract from acetone-dried pig or beef liver. The preparation of Dewan (2) was the precipitate obtained at pH 4.6 from an extract of acetone-dried pig liver or kidney. Both studies showed approximately the same results. The preparations with L-glutamate and diphosphopyridine nucleotide (DPN) reduced methylene blue or took up oxygen when a suitable carrier was present. The final reaction products were shown to be ammonia and α -ketoglutarate with α -iminoglutarate as an intermediate product. The reaction was found to be reversible and the enzyme in question was shown to be highly specific for L-glutamic acid.

The present paper deals with the adaptation of the known facts of this enzyme system to tissue homogenates.

EXPERIMENTAL

Material and Methods—The animal tissues used in this study were obtained from young male rats of the Holtzman strain, approximately 3 to 4 months of age. The rats were killed by cervical dislocation and the tissue sample quickly taken, blotted, weighed, and placed in a cold homogenizing tube containing 0.5 ml. of water. The tissue was homogenized and sufficient cold glass-distilled water was added to give a 5 per cent homogenate. Aliquots of the homogenate were added to the properly fortified substrate in Warburg flasks with no side arms, which were kept cold until the tissue was added. The cold flasks were then attached to the manometers and transferred to the 38° bath and the measurement of oxygen uptake was begun after a 6 minute equilibration period.

The glutamic acid and semicarbazide hydrochloride were obtained from the Eastman Kodak Company. The latter was further purified by dissolving in hot water and recrystallizing with the addition of 2 to 3 volumes

*This investigation was supported in part by a grant from the Committee for Research in Endocrinology of the National Research Council, and in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

of cold alcohol. Cytochrome c was prepared in this laboratory according to the method of Keilin and Hartree (3) and was standardized photometrically (4). The DPN was prepared according to the method of LePage (5) and was 72 per cent pure as determined by spectrophotometric analysis. We are indebted to Dr. G. A. LePage for a sample of 85 per cent pure triphosphopyridine nucleotide (TPN) which was used in this study. All reagents, except cytochrome, were employed as the neutral potassium salts.

Table I
Optimum Conditions for Glutamic Dehydrogenase System

The source of enzyme was rat liver; 0.2 ml. of a 5 per cent water homogenate was added to each flask. The data shown for each compound added were the average of two experiments with a freshly prepared homogenate. Each compound was studied at varying concentrations with all other compounds held constant and at the optimum value. The concentration of each component selected for the assay technique is indicated by bold-faced type. The reaction volume of each flask was 30 ml. The $Q_{\rm O_2}$ was calculated on the basis of the two best consecutive 10 minute periods.

Glutamate		Phosphate		H+ ions		DPN		rome c	Semicai	rbazıde
Q_{02}	Molarity	Q ₀₂	pH	Q ₀₂	Mg. per flask	Q ₀₂	Molar- ity X 10-5	Q _o .	Molar-	Q ₀₂
4.0	0	50.6	6.0	16.0	0	0	0	8.0	0	43.1
41.2	0.0033	51.3	6.6	33.3	2	20.4	0.65	28.9	0.025	40.9
47.3	0.01	49.0	7.0	42.2	4	38.6	1.32	49.1	0.05	43.6
49.7	0.02	47.8	7.2	43.0	6	50.4	2.64	48.9	0.075	49.9
51.2	0.027	48.3	7.4	41.6	8	50.0	4.00	49.2	0.100	48.2
48.2	0.04	43.4	8.0	31.7			5.32	48.1		
	Q ₀₂ 4.0 41.2 47.3 49.7 51.2	4.0 0 41.2 0.0033 47.3 0.01 49.7 0.02 51.2 0.027	Q _{O2} Molarity Q _{O2} 4.0 0 50.6 41.2 0.0033 51.3 47.3 0.01 49.0 49.7 0.02 47.8 51.2 0.027 48.3	Q ₀₂ Molarity Q ₀₂ pH 4.0 0 50.6 6.0 41.2 0.0033 51.3 6.6 47.3 0.01 49.0 7.0 49.7 0.02 47.8 7.2 51.2 0.027 48.3 7.4	Q _{O2} Molarity Q _{O2} pH Q _{O2} 4.0 0 50.6 6.0 16.0 41.2 0.0033 51.3 6.6 33.3 47.3 0.01 49.0 7.0 42.2 49.7 0.02 47.8 7.2 43.0 51.2 0.027 48.3 7.4 41.6	Q ₀₂ Molarity Q ₀₂ pH Q ₀₂ Mg. per flask 4.0 0 50.6 6.0 16.0 0 41.2 0.0033 51.3 6.6 33.3 2 47.3 0.01 49.0 7.0 42.2 4 49.7 0.02 47.8 7.2 43.0 6 51.2 0.027 48.3 7.4 41.6 8	Q _{O2} Molarity Q _{O2} pH Q _{O2} Mg. per flask Q _{O2} 4.0 0 50.6 6.0 16.0 0 0 41.2 0.0033 51.3 6.6 33.3 2 20.4 47.3 0.01 49.0 7.0 42.2 4 38.6 49.7 0.02 47.8 7.2 43.0 6 50.4 51.2 0.027 48.3 7.4 41.6 8 50.0	Q_{02} Molarity Q_{02} pH Q_{02} $M_{g. per}$ flask Q_{02} $M_{olarity}$ flask 4.0 0 50.6 6.0 16.0 0 0 0 41.2 0.0033 51.3 6.6 33.3 2 20.4 0.65 47.3 0.01 49.0 7.0 42.2 4 38.6 1.32 49.7 0.02 47.8 7.2 43.0 6 50.4 2.64 51.2 0.027 48.3 7.4 41.6 8 50.0 4.00	$\begin{array}{ c c c c c c c c c c c c c c c c c c c$	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$

Water versus Isotonic Homogenates—In previous studies, water homogenates have been used successfully in the determination of the succinic dehydrogenase and cytochrome oxidase system (6), of the glycolytic system (7), and of the malic system (8). On the other hand, isotonic homogenates have been demonstrated to be superior to water homogenates in the oxidation of oxalacetate (9) and the oxidation of octanoate (10). The average Q values of two representative determinations of glutamic dehydrogenase were 48.3 for water homogenates and 36.1 for isotonic homogenates. On this basis, water homogenates were used throughout this study.

Components of System—The study of the optimum concentrations of the various components of this system is summarized in Table I. The optimum pH was found to be 7.3 as reported by Dewan (2), who used pyocyanine as the carrier. The system does not appear to need additional

sources of inorganic phosphate, but an appreciable amount was used in order to increase the buffering capacity of the medium. The data demonstrate the need for each of the other components, and the effect of each can be explained on the basis of the accompanying diagram in which Reactions A, B, and C are catalyzed by enzymes which are furnished by the homogenate, and are considered to represent glutamic dehydrogenase, DPN-cytochrome c reductase, and cytochrome oxidase respectively.

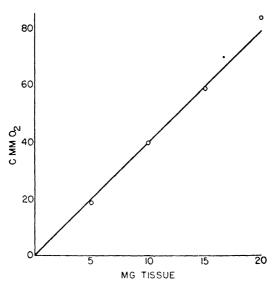
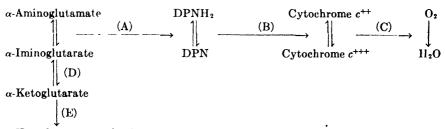


Fig. 1. Effects of different amounts of liver homogenate on oxygen consumption during a 20 minute period. The complete reaction mixture is as described in the text.



α-Ketoglutarate semicarbazone

Enzymes in the homogenate determine the rate of oxygen consumption when the critical components listed in Table I are present in excess. Proof that this is true is presented in Fig. 1, in which the rate is shown to be proportional to the amount of homogenate added. If one of the enzymes is definitely the limiting component, Fig. 1 shows that the system is probably

valid for assay work. Previous assays for DPN-cytochrome c reductase (8) and cytochrome oxidase (6) show that these enzymes are present in rat liver in larger amounts than required here and are not limiting. Thus glutamic dehydrogenase appears to be the limiting enzyme in the system described in this paper.

The addition of nicotinamide at various concentrations to the reaction mixture had no beneficial effects on the enzyme system under study. The inclusion of semicarbazide as one of the components of this system is of questionable necessity, as shown in Table I. Dewan (2) has shown, however, that semicarbazide aids in maintaining the rate of reaction over a period of time. Our results confirm this observation. The theoretical need for the semicarbazide is apparent in the light of the results of von Euler et al. (1) which show that the equilibrium between α -amino- and α-iminoglutarate markedly favors the former. They also showed that significant concentrations of either ammonia or α -ketoglutarate, or small concentrations of both, inhibited the reduction of DPN by glutamic dehydrogenase. Therefore, the removal of one of the products of the oxidative deamination of glutamate by means of Reaction E will allow Reaction D, which is non-enzymatic, to proceed toward the formation of the keto acid. Thus, the removal of the keto acid tends to overcome the inhibitory effect of the unfavorable equilibrium on the dehydrogenation of glutamate. The semicarbazide also prevents the further metabolism of the a-ketoglutarate. This gives assurance that the oxygen consumption of the reconstituted system is a measure of the dehydrogenation of glutamate only and not the sum of this step plus the oxidative steps leading from α -ketoglutarate.

That the equilibrium between the amino and imino acids favors the formation rather than the breakdown of the amino acid may, in part, explain why such large quantities of DPN are necessary for optimum activity. It is apparent that the fixation of the end-product, the high substrate concentration, and the high concentration of DPN are all necessary to drive Reaction A in the desired direction at the maximum velocity.

DPN versus TPN—The two earlier studies (1, 2) both demonstrated that glutamic dehydrogenase is a coenzyme-linked enzyme. Both laboratories showed that DPN could serve as the coenzyme for the oxidation of glutamate. Neither group was able to demonstrate the oxidation of glutamate with TPN (2, 11) although it has been shown (1) that reduced TPN, as well as reduced DPN, readily facilitates the formation of glutamate from ammonia and α -ketoglutarate. A comparison of the effectiveness of the two pyridine nucleotides in the enzyme system studied here is shown

in Table II. The data clearly indicate that TPN is less than 10 per cent as effective as DPN at comparable concentrations.

Recently, Mehler, Kornberg, Grisolia, and Ochoa (12) demonstrated that TPN and DPN worked equally well as the coenzyme for glutamic dehydrogenase. These workers used the spectrophotometric reduction of the pyridine nucleotide as the measure of activity. In this laboratory, with whole homogenates as the enzyme source, the spectrophotometric

TABLE II

Comparison of TPN and DPN

Source of enzyme, 0.2 ml. of a 5 per cent rat liver homogenate. The amount of coenzyme indicated is calculated as pure coenzyme. All the conditions are optimum except for the coenzyme. The data are expressed as Q_{02} .

Pyridine nucleotide	DPN	TPN
mg. per flask		
1.5	22.8	0.0
3.0	46.0	1.5
4.5	59.3	5.1

TABLE III
Glutamic Dehydrogenase Assay of Various Rat Tissues

The tissues were prepared for study in the form of 5 per cent water homogenates and were added at a level of 10 mg. of tissue per flask, with all conditions optimum. The data are expressed in terms of Q_{0_1} .

Tissue	No. of samples	Glutamic dehydrogenase, average and range
Liver	43	49.3 (37.2-62.6)
Kidney	3	24.7 (19.3-29.1)
Heart	3	4.9 (0.0-8.0)
Spleen	2	5.2 (3.5-6.8)
Brain	3	10.3 (6.2-15.5)

determination of the reduced coenzyme likewise showed that both pyridine nucleotides will react equally well with glutamic dehydrogenase. Thus, it is evident that the inability of TPN to serve as a coenzyme in the manometric determination of glutamic dehydrogenase is not due to a coenzyme specificity of the dehydrogenase but rather to an inability of reduced TPN to link with the cytochrome system. Horecker (13) has recently isolated a purified TPN-cytochrome c reductase from pig liver. Such an enzyme, if present in the rat liver used in these studies, was not present in sufficient

concentration in the dilute homogenate to be effective in linking reduced TPN to the cytochrome c to any noticeable extent.

Assay Method and Results—On the basis of the foregoing studies the optimum reaction medium finally selected for the assay of glutamic dehydrogenase consists of the following: water to make a final volume of 3.0 ml., 0.3 ml. of 0.2 m potassium phosphate (pH 7.2), 0.6 ml. of 0.5 m potassium glutamate, 0.6 ml. of 1 per cent K-DPN (72 per cent pure), 0.2 ml. of 4×10^{-4} m cytochrome c, 0.2 ml. of 1.0 m semicarbazide (added just before the tissue), and 0.2 ml. of a 5 per cent water homogenate. The center well contained 0.1 ml. of 10 per cent KOH.

A number of assays have been carried out on rat liver and the results, together with data from other rat tissues, are presented in Table III. The relative activities of the various tissues compare closely with those obtained by von Euler et al. (1) from extracts of rat tissues. Arbitrarily assigning liver the activity of 100, these workers found the activity ratio of liver to kidney to brain to be 100:41:6. With the method outlined in the present study, the activity ratio for these three tissues was found to be 100:50: 21. The demonstrable activity of brain homogenates again confirms the findings of Weil-Malherbe (14) and subsequent workers that brain tissue possesses the ability to metabolize glutamic acid.

SUMMARY

- 1. A method has been described for the assay of glutamic dehydrogenase in animal tissues.
- 2. The proposed technique is based on the measurement of the rate of oxygen uptake of water homogenates in a properly fortified medium by use of the conventional Warburg apparatus.
- 3. It has been demonstrated that DPN and not TPN is the necessary coenzyme for the manometric method proposed here. It is probable that the lack of oxygen uptake in the presence of TPN is due to a lack of TPN-cytochrome c reductase and not to a coenzyme specificity of the dehydrogenase, since spectrophotometric determinations have demonstrated the reduction of TPN by glutamic dehydrogenase.
 - 4. Assays of various rat tissues are reported.

BIBLIOGRAPHY

- von Euler, H., Adler, E., Günther, G., and Das, N. B., Z. physiol. Chem., 254, 61 (1938).
- 2. Dewan, J. G., Biochem. J., 32, 1378 (1938).
- 3. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 122, 298 (1937).
- Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 188 (1945).

- 5. LePage, G. A., J. Biol. Chem., 168, 623 (1947).
- 6. Schneider, W. C., and Potter, V. R., J. Biol. Chem., 149, 217 (1943).
- 7. Novikoff, A. B., Potter, V. R., and LePage, G. A., J. Biol. Chem., 173, 239 (1948).
- 8. Potter, V. R., J. Biol. Chem., 165, 311 (1946).
- 9. Potter, V. R., LePage, G. A., and Klug, H. L., J. Biol. Chem., 175, 619 (1948).
- 10. Lehninger, A. L., J. Biol. Chem., 161, 437 (1945).
- 11. Adler, E., Das, N. B., von Euler, H., and Heyman, Y., Compt.-rend. trav. Lab. Carlsberg. 22, 15 (1938).
- Mehler, A. H., Kornberg, A., Grisolia S., and Ochoa, S., J. Biol. Chem., 174, 961 (1948).
- 13. Horecker, B. L., Federation Proc., 8, 207 (1949).
- 14. Weil-Malherbe, H., Biochem. J., 30, 665 (1936).

THE DIABETOGENIC EFFECT OF DEHYDROASCORBIC AND DEHYDROISOASCORBIC ACIDS*

By JOHN W. PATTERSON

(From the Deq rtment of Anatomy, School of Medicine, Western Reserve University, Cleveland, Ohio)

(Received for publication, August 26, 1949)

Ascorbic acid is known to exist in the animal body in a reversibly oxidized form as dehydroascorbic acid (I). Its chemical structure in the non-hydrated state is similar to that of alloxan (II).

The chemical properties of these two substances are strikingly similar. Both compounds are irreversibly decomposed under biological conditions. Alloxan has a half life of 1 minute (1) and dehydroascorbic acid has a half life of a few minutes (2). Alloxan is readily reduced to dialuric acid (3) with which it forms alloxantin (3). Dehydroascorbic acid is readily reduced to ascorbic acid (4) and also forms a similar intermediate compound (5). Alloxan (6) and dehydroascorbic acid (7) both form addition compounds with molecules containing the sulfhydryl group. Alloxan (8) and dehydroascorbic acid (9) both produce the Strecker reaction with amino acids and give the ordinary ketone reactions.

It would appear logical, therefore, to expect that there might be a relationship between alloxan and dehydroascorbic acid with regard to the production of diabetes. There is some evidence suggesting that dehydroascorbic acid may have diabetogenic properties. It is known that the dehydroascorbic acid level in the scorbutic animal is higher than normal, the dehydroascorbic acid-ascorbic acid ratio being as much as 20 times the normal value (10). Correlated with this is the fact that scorbutic guinea pigs have a decreased glucose tolerance (11) and show degeneration of the islets of Langerhans (12). Furthermore, ascorbic acid is known to act synergistically with alloxan (13, 14) and it seems probable that this effect

^{*} Aided in part by a grant from the Cleveland Diabetic Fund.

is the result of the formation of dehydroascorbic acid. This is shown in the present work. It is also demonstrated that dehydroascorbic and dehydroisoascorbic acids produce diabetes in a manner similar to alloxan.

EXPERIMENTAL

Reagents---

Alloxan (Eastman).

Crystalline ascorbic Acid (Merck).

Dehydroascorbic acid was prepared immediately before each series of injections by dissolving ascorbic acid (1.08 gm.) in water (10 cc.) and shaking for 15 minutes with an equal volume of ether containing freshly sublimed quinone (0.66 gm.) (15). The ether layer was removed and the water solution was washed five times with an equal volume of ether. The excess ether was removed by suction and the solution used without further treatment. The yield is known to be excellent (16) and the solution was assumed to have about 100 mg. of dehydroascorbic acid per cc. The product was free of ascorbic acid by spectrophotometric examination and contained from 95 to 98 mg. of dehydroascorbic acid per cc. by polarimetric analysis. Slow decomposition produces a product with the opposite sign of rotation; thus these values are probably slightly low (17).

Dehydroisoascorbic acid was prepared from isoascorbic acid¹ by the procedure described above.

Action of Dehydroascorbic Acid on Rats—In male rats weighing 100 to 150 gm. the injection of 10 to 50 mg. of dehydroascorbic acid produces a characteristic reaction. Immediately after intravenous injection the rat becomes excited and runs aimlessly but rapidly around the cage for about 2 minutes. From time to time during this period, it may raise itself on its hind legs and vigorously rub its face with its fore legs. After this period of hyperactivity the rat collapses. It gradually starts to gasp for breath and slowly resumes normal respiration. There may be a slight serous discharge from the nose and mouth during the period of prostration. There is usually a brownish red discharge apparent around the eyes. With a dose of 20 mg. the animals appear normal about 10 minutes after the injection.

With larger doses the rats may not recover after the collapse. In a series of male Sprague-Dawley rats, 116 to 124 gm. in weight, that were injected intravenously with dehydroascorbic acid, there were no deaths in seven given 20 mg., one death in four given 30 mg., four deaths in eight given 40 mg., and four deaths in six given 50 mg. In the rats that died the heart continued to beat after respiration had ceased. At autopsy it was noted that

¹ Kindly donated by Dr. H. G. Luther of Chas. Pfizer and Company, Inc.

there was no frothing in the trachea, that a section of lung would float in water, and that the right ventricle of the heart was dilated with blood, whereas the left ventricle was small.

The characteristic hyperactive reaction could be produced with dehydroascorbic acid prepared by the oxidation of ascorbic acid with sodium hypochlorite, indicating that the effect was not due to an organic contaminant.

It is of great interest to note that the rats which had recovered from one injection of dehydroascorbic acid immediately tolerated a second dose several times the size of the initial dose, up to a maximum of about 1 gm. per This increased tolerance was noted as early as 10 minutes and as late as several hours after the administration of the initial dose. After a large second dose it was possible to give a similar large dose as much as a week later without killing the animal. The exact limits of this tolerance have not yet been determined. When the initial dose of dehydroascorbic acid was relatively large, there was little or no hyperactive response following the second dose. The rats became lethargic but occasionally they would bite an adjacent rat or object. With large doses respiration frequently became difficult. In those rats that died the autopsy findings were the same as those in rats that died following the initial dose. When the initial dose of dehydroascorbic acid was small, there was a second hyperactive response following the second dose. A small initial dose would not permit the administration of a large second dose.

Dehydroisoascorbic acid produced a similar hyperactive reaction in the rat, but a larger dose was required to produce the same effect and the lethal dose was much higher. As much as 160 mg. could be given as an initial dose with few deaths occurring. With this substance the animals did not tolerate a second dose that was greatly different from a large initial dose.

Synergistic Action of Dehydroascorbic and Ascorbic Acids with Alloxan—Male rats of Albino Farms stock were injected intravenously with alloxan without previous fasting and then, by the same needle, with ascorbic acid or dehydroascorbic acid. Diabetes was determined by the 48 hour blood sugar as in previous ascorbic acid studies (13, 14). Blood sugars over 150 mg. per cent were considered an indication of diabetes. When ascorbic acid was given with the alloxan, an alloxan dose of 20 mg. per kilo was necessary to produce diabetes in 44 per cent of the rats, whereas when dehydroascorbic acid was given with alloxan, an alloxan dose of 15 mg. per kilo was sufficient to produce diabetes in 58 per cent of the rats (Table I). Ascorbic acid with this smaller dose of alloxan had no effect on the level of blood sugar.

Diabetogenic Action of Dehydroascorbic and Dehydroisoascorbic Acids-

Blood sugars of male Sprague-Dawley rats were measured by a micromethod (18) before and from 2 to 14 days after intravenous injection of the test substances (Table II). The maximum dose of dehydroascorbic acid

Table I

Synergistic Action of Ascorbic Acid and Dehydroascorbic Acid with Alloxan after

Intravenous Administration in Male Rats

	Weig	Weight		Dehydro- ascorbic acid	Ascorbic acid	Dia	betic	Non-diabetic		
No. of rats Range	Average	Alloxan	No. of rats			Average 48 hr. blood sugar	No. of	Average 48 hr. blood sugar		
	gm.	gm.	mg. per kg.	mg.	mg.		mg. per 100 cc.		mg. per 100 cc.	
6	102-154	117	30	20		6	392			
4	120-158	130	30		20	4	334			
4	96-144	112	20	20	ļ	4	375			
9	100-134	121	20		20	3	190	6	126	
12	96-118	106	15	15		7	242	5	113	
12	88-116	104	15		15			12	118	
2	98-108	103	10	15				2	108	
2	102	102	10		20			2	122	
7	138-156	150	0	20				7	126	

Table II

Diabetogenic Action of Dehydroascorbic and Dehydroisoascorbic Acids after

Intravenous Administration in Male Rats

No. of	0. 01		Dehydro- isoascor-		Average	e blood		efore a er 100 c		r inje	ction,				
rats	Range	Aver- age	acid 1	acid	Aver-	bic acid	0 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	14 days
	gm.	gm.	gm. per kg.	gm. per kg.											
4*	116-120	118	1.1		110	125	330	182	156	132	130	133			
4	130-138	133		1.0	138	327	312	208	127	107	141	137			
2	130	130		1.5	137	400	450	450	45 0	450	450	450	450		
4	120-126	123		1.4	116	246		357			371		327		
2†	150-163	156		1.5	104	332		400			425		450		

^{* 40} mg. were given each rat as a preliminary dose 10 to 30 minutes before the indicated dose.

that could be given after an initial small dose, administered 10 to 30 minutes earlier, was about 1.1 gm. per kilo. In the rats that survived the injections hyperglycemia developed on the 3rd day and persisted for 3 days. 1 gm. per kilo of dehydroisoascorbic acid produced essentially the

[†] Fasted overnight.

same temporary hyperglycemia as did dehydroascorbic acid. The injection of 1.4 gm. per kilo or more of dehydroisoascorbic acid produced a permanent diabetes. One rat, observed for 4 months, maintained a blood sugar of over 400 mg. per cent and four rats showed a similar high blood sugar level after 6 weeks. A number of rats with hyperglycemia died within 2 weeks; one died on the 17th day and two after 6 weeks. Terminally, the blood sugar values tended to drop.

The effect of repeated doses of dehydroascorbic acid is shown in Table III. On the 1st day an initial dose of 20 mg. was administered intravenously 10 to 30 minutes before the first of three or more daily intravenous injections of 80 mg. Mortality during this period of injection was about 25 per cent. Of fourteen rats surviving a series of three or four injections,

Table III

Diabetogenic Action of Repeated Intravenous Injections of Dehydroascorbic Acid in

Male Rats

Weight	Dehydro- ascorbic	No. of daily									
of rat	of rat acid*	injections	Initial	2-3 days	4-5 days	6-7 days	14 days	21 days			
gm.	mg. per day										
119	60-80	10	148	450	400	410	310	375			
112	80	3	129	450	450	450	450	450			
149	80	3	127	435	380	315	400	176			
113	80	3	87	450	140	185	113	100			
127	80	3	123				390	395			
126	80	3	145				280	270			
128	80	4	141	450	450	450	450	450			
154	80	4	114	430	330	355	257	108			

^{*} On the 1st day an initial dose of 20 mg. was injected intravenously 10 to 30 minutes before the indicated dose.

seven died with elevated blood sugars within 3 weeks, the highest mortality occurring about a week after the completion of the injections. The results on seven surviving rats along with one that received more injections are reported in Table III. At the end of 3 weeks there was a marked hyperglycemia in five of the eight rats. Smaller doses produced less striking results. However, one rat that received 60 mg. daily for 9 days showed an irregular hyperglycemia and a marked decrease in tolerance to glucose 40 days after the last injection. The intraperitoneal administration of 3.5 gm. of glucose per kilo produced an immediate rise in blood sugar which was maintained above 450 mg. per cent for 3 hours. The hyperglycemia resulting from daily injections of dehydroascorbic acid responded to the injection of small amounts of insulin.

DISCUSSION

A characteristic hyperactive reaction is produced in the rat by the intravenous injection of dehydroascorbic acid, and to a lesser degree by the injection of dehydroisoascorbic acid. Dehydroisoascorbic acid results from the oxidation of isoascorbic acid, which is a synthetic analogue of ascorbic acid and differs from it only in that it has the opposite optical configuration on carbon atom 5. Isoascorbic acid has only one-twentieth of the antiscorbutic activity of ascorbic acid (19). Therefore, the fact that dehydroisoascorbic acid has less tendency to produce the hyperactive response of dehydroascorbic acid may indicate that the reaction occurring with dehydroascorbic acid is merely an exaggeration of a normal biochemical process.

There is an increased tolerance to a second dose of dehydroascorbic acid with a marked decrease in the characteristic hyperactive reaction that follows the initial dose. The increased tolerance is noted 10 minutes after the initial dose and is prolonged. The fact that a large second dose of dehydroascorbic acid produces less hyperactivity than a smaller initial dose seems to indicate that the hyperactive response is mediated by a substance liberated in vivo which is present in a limited quantity. Thus, if all of this substance is liberated at one time, death results, while liberation of most of it causes the characteristic reaction without death. The second dose then merely releases the small amount remaining without causing any effect. It is interesting that some of the effects produced by dehydroascorbic acid are similar to those produced by acetylcholine.

It seems likely that toxic manifestations previously reported for ascorbic acid (20) may have been due to the presence of dehydroascorbic acid.

Dehydroascorbic acid under the conditions used is more effective than ascorbic acid in acting synergistically with alloxan to produce diabetes. It is possible that the effect of ascorbic acid is the result of its conversion to dehydroascorbic acid by the oxyhemoglobin (21) of the blood which is released on hemolysis following the injection of alloxan (22). Dehydroascorbic acid possibly acts by lowering glutathione and thus having a sparing action on alloxan (23).

Permanent hyperglycemia is produced by the injection of 1.4 gm. per kilo of dehydroisoascorbic acid. In a dose of 1 gm. per kilo, a temporary diabetes is produced. In this same dosage dehydroascorbic acid produces essentially the same result as dehydroisoascorbic acid. The general toxicity of dehydroascorbic acid prevents the use of a higher dosage. However, repeated injections of dehydroascorbic acid seem to be capable of producing a permanent diabetes. It is believed that this is the first time that diabetes, seemingly permanent, has been produced by a known chemi-

cal substance that is not closely related to alloxan. The fact that dehydro-ascorbic acid occurs physiologically in man and is capable of producing permanent diabetes is also significant.

There does not appear to be a correlation between the hyperactive response following the intravenous injection of these compounds and their diabetogenic effect. On the basis of the temporary hyperglycemia produced with smaller doses, dehydroascorbic acid and dehydroisoascorbic acid seem to have about the same potency as far as the production of diabetes is concerned. However, dehydroascorbic acid is 4 or more times as effective as dehydroisoascorbic acid in producing a hyperactive response.

The mechanism of action of these substances is probably similar to that of alloxan (13, 24) which is thought to interfere with essential sulf-hydryl enzymes of the β cells. The similarity in action to that of alloxan is indicated by the previously demonstrated (25) triphasic glucose response following the injection of dehydroascorbic acid. The action on the β cells with decreased insulin production is indicated by the ready response of the hyperglycemia to injected insulin.

It is too early to generalize about the essential chemical structure required for the production of diabetes, but the results with dehydroascorbic and dehydroisoascorbic acids lead to a greater variation in possible structure. In searching for a precipitating factor in human diabetes, it becomes necessary to broaden the search beyond the nitrogen-containing purines and pyrimidines to substances which may be derived from carbohydrates, and as a matter of fact to any substance that may cause an excessive oxidation of ascorbic acid to dehydroascorbic acid in the β cells of the islets of Langerhans.

SUMMARY

- 1. Dehydroascorbic acid produces a characteristic reaction in rats with an LD₅₀ of about 320 mg. per kilo. Following a sublethal dose, rats will tolerate a second dose 3 to 4 times as large as the initial dose.
- 2. Dehydroascorbic acid is more effective than ascorbic acid in acting synergistically with alloxan to produce diabetes.
- 3. Dehydroascorbic acid is shown to be similar to alloxan in structure and chemical properties, and to produce a hyperglycemia of a few days duration following a dose of 1.1 gm. per kilo in rats. Three daily injections of 80 mg. are capable of producing what appears to be permanent diabetes in rats weighing about 120 gm.
- 4. Dehydroisoascorbic acid produces permanent diabetes in doses of 1.5 gm. per kilo.

BIBLIOGRAPHY

- 1. Patterson, J. W., Lazarow, A., and Levey, S., J. Biol. Chem., 177, 187 (1949).
- Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., J. Biol. Chem., 117, 237 (1937).
- 3. Archibald, R. M., J. Biol. Chem., 158, 347 (1945).
- 4. Hopkins, F. G., and Morgan, E. J., Biochem. J., 30, 1446 (1936).
- 5. Bezssonoff, M. N., and Woloszyn, M., Bull. Soc. chim. biol., 20, 93 (1938).
- 6. Patterson, J. W., Lazarow, A., and Levey, S., J. Biol. Chem., 177, 197 (1949).
- 7. Drake, B. B., Smythe, C. V., and King, C. G., J. Biol. Chem., 143, 89 (1942).
- 8. Strecker, A., Ann. Chem., 123, 363 (1862).
- 9. Abderhalden, E., Fermentforschung, 15, 522 (1938).
- 10. Bonsignore, A., and Pinotti, F., Quaderni nutriz., 2, 333 (1935).
- 11. Sigal, A., and King, C. G., J. Biol. Chem., 116, 489 (1936).
- 12. Banerjee, S., Nature, 153, 344 (1944).
- 13. Lazarow, A., Proc. Soc. Exp. Biol. and Med., 61, 441 (1946).
- 14. Levey, S., and Suter, B., Proc. Soc. Exp. Biol. and Med., 63, 341 (1946).
- 15. Moll, T., and Wieters, H., E. Merck's Jahresber., 50, 65 (1936).
- 16. Schultze, M. O., Harrer, C. J., and King, C. G., J. Biol. Chem., 131, 5 (1939).
- Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., J. Chem. Soc., 1270 (1933).
- 18. Folin, O., and Malmros, H., J. Biol. Chem., 83, 115 (1929).
- 19. King, C. G., Physiol. Rev., 16, 238 (1936).
- 20. Widenbauer, F., Klin. Wochschr., 15, 1158 (1936).
- 21. Vladimirov, G. E., and Kolotilova, A. I., Biokhimiya, 12, 321 (1947).
- 22. György, P., and Rose, C. S., Science, 108, 716 (1948).
- 23. Lazarow, A., Physiol. Rev., 29, 48 (1949).
- 24. Lazarow, A., Patterson, J. W., and Levey, S., Science, 108, 308 (1948).
- 25. Patterson, J. W., Endocrinology, 45, 344 (1949).

A PARTIAL SEPARATION AND CHARACTERIZATION OF CYTOCHROME OXIDASE AND CYTOCHROME b*

By BERTRAM EICHEL, W. W. WAINIO, and P. PERSON (From the Bureau of Biological Research, Rutgers University, New Brunswick)

AND S. J. COOPERSTEIN

(From the Department of Anatomy, School of Medicine, Western Reserve
University, Cleveland)

(Received for publication, September 15, 1949)

Numerous attempts have been made to separate the insoluble cytochromes of mammalian heart muscle. Von Euler and Hellström in 1939 (1) used sodium cholate and ammonium sulfate and obtained a preparation containing only cytochrome b. Straub (2) obtained a fraction with sodium cholate and ammonium sulfate in which he demonstrated cytochrome oxidase (called cytochrome a_3 by him after Keilin and Hartree (3)) and very little cytochrome b. In the same year Yakushiji and Okunuki (4), also using sodium cholate and ammonium sulfate, claimed the preparation of cytochrome oxidase (called cytochrome a by them). To us the preferential solubility in sodium desoxycholate of proteins that are inactive when tested with the hydroquinone-cytochrome c system (5, 6) suggested that the various components of the insoluble complex might be separated by successive additions of small amounts of desoxycholate (7).

The characterization of cytochrome oxidase and cytochrome b has rested principally on a knowledge of their absorption spectra. Keilin and Hartree (3) found bands at 600 m μ and 448 m μ for reduced cytochrome a_3 (which they suggest may be oxidase), at 605 m μ and 452 m μ for reduced cytochrome a (the existence of which we do not consider proved), and at 564, 530, and 432 m μ for reduced cytochrome b. Keilin and Hartree (3) and Graubard (8) have suggested that cytochrome oxidase may be a copper-containing enzyme.

This report deals with the details of a partial separation and purification of the cytochromes contained in an insoluble preparation of mammalian heart muscle. Cytochrome b and cytochrome oxidase are characterized with respect to their absorption spectra in both the oxidized and reduced

This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service. The first part of this work was done in the Department of Physiology at the New York University College of Dentistry and was supported by a grant from the John and Mary R. Markle Foundation.

^{*} With the assistance of Ruthanna Hoopes and Herbert J. Eichel.

states. Evidence is presented which indicates that cytochrome oxidase is a copper-containing enzyme.

EXPERIMENTAL

The insoluble cytochrome complex preparation was obtained from beef heart and was a suspension precipitated one or three times by acetate as previously described (9, 6).

The test of enzyme activity was adapted from the methods of Haas (10), Keilin and Hartree (11), and Stotz, Sidwell, and Hogness (12). The gas phase was air and the temperature 25°. Each vessel contained 3.0 mg. of hydroquinone in 0.25 ml. of water (side arm), 1.0 mg. of cytochrome c in 0.25 ml. of water, 0.3 ml. of 0.1 m semicarbazide-HCl (previously adjusted to pH 7.1 with NaOH), 1.45 ml. of 0.1 m Na₂HPO₄-KH₂PO₄ buffer of pH 7.1, 0.25, 0.5, or 0.75 ml. of the enzyme preparation, and distilled water, when necessary, to make the total volume 3.0 ml. The limit of the oxygen uptake by this system is approximately 90 c.mm. in 15 minutes. 1 ml. of the insoluble preparation or 1 "ml. equivalent" of the lyophilized enzyme preparations was dissolved in 25 ml. of cold distilled water for the test.

Copper was determined by the method of McFarlane (13), iron by the method of Lintzel (14), and protein by the micro-Kjeldahl method by use of the factor 6.25 to convert nitrogen to protein.

Insoluble Complex—The insoluble preparations made from beef heart, when tested manometrically with the hydroquinone-cytochrome c system, gave $Q_{\rm o_2}$ protein values at 25° ranging from 260 to 600. A typical preparation, the one from which the preparation next described was prepared, had a $Q_{\rm o_2}$ protein value of 300.

Preparation 0-4—The addition of 40 mg. of sodium desoxycholate² (4 per cent) to each ml. of the cold insoluble preparation (precipitated three times by acetate), followed by grinding in a cold mortar, and by centrifugation for 1 hour at $25,000 \times g$,³ yields a slightly opalescent, red-brown supernatant. The solution may be lyophilized. Approximately 80 per cent of the protein is dissolved, and the $Q_{\rm O_2}$ protein values at 25° range from 1140 to 1840. A preparation made from the insoluble complex discussed in the preceding section had a $Q_{\rm O_2}$ protein of 1840. The observed density values⁴ of a solution made by dissolving 1 ml. equivalent in 20 ml. of distilled water were divided by the mg. of protein in the solu-

¹ 1 "ml. equivalent" represents the amount of dry preparation obtained by lyophilizing 1 ml. of the concentrated supernatant.

² Difco

³ International multispeed attachment, six place head, 10 ml. Lusteroid tubes.

⁴ Beckman spectrophotometer.

tion. This is justified on the basis of the relationship, K = D/CL, where K is a constant, D is the density, C is the concentration of protein (expressed here in mg.), and L is the length of the solution absorbing the light (1 cm. here). The calculated values plotted against the wavelengths are given in Fig. 1. The curve for the oxidized system shows a peak at 414 m μ . The curve for the reduced⁵ system has a peak at 428 m μ (cytochrome b) with a slight skewing at 415 to 420 m μ (cytochrome

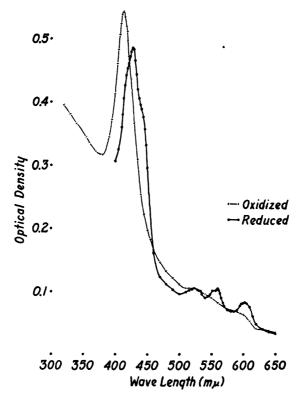


Fig. 1. Preparation 0-4

c) and a marked skewing at about 442 m μ (cytochrome oxidase). The hump with a maximum at 525 m μ and the hump with components at 560 and 550 m μ belong to cytochrome b and cytochrome c. The 602 m μ peak belongs to cytochrome oxidase. There is evidence, therefore, for the presence of the cytochromes b, c, and oxidase in this preparation.

⁵ Reduced with a pinch of sodium hydrosulfite. The absorption by the reduced enzymes cannot be analyzed below 400 m μ because of the marked absorption by sodium hydrosulfite in this region.

Flavoprotein does not exhibit its 450 m μ peak on the curve of the oxidized system.

Preparations 0-2 and 2-3—The partial purification of cytochrome oxidase, by the use of sodium desoxycholate, has been previously described (6). The same procedure can be made to yield an almost complete separation of the cytochromes b and oxidase. If to each ml. of the cold insoluble preparation (precipitated three times by acetate) one adds 20 mg. (2 per cent) of sodium desoxycholate in a cold mortar with grinding or in a beaker with stirring and centrifuges the mixture in the cold at 25,000 \times g for 1 hour, the supernatant (termed Preparation 0-2) contains mainly

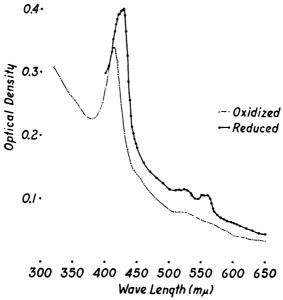


Fig. 2. Preparation 0-2

cytochrome c and cytochrome b and only small amounts of cytochrome oxidase. The supernatant can be lyophilized to yield a dry preparation. The Q_{02} protein (25°) values of the lyophilized preparations range from 25 to 260; the preparation described here has a value of 230. The absorption spectrum (Fig. 2) of this preparation, made by dissolving 1 ml. equivalent in 10 ml. of cold distilled water is expressed in terms of optical density per mg. of protein (K = D/CL), and shows a peak at about 413 m μ in the oxidized state. The curve for the reduced components has a peak at 428 m μ (cytochrome b) which is markedly skewed toward the blue by a component absorbing at about 415 to 420 m μ (cytochrome c).

The hump with a maximum at 522 m μ and that with a plateau at 550 to 560 m μ belong to cytochrome c and cytochrome b. Sometimes, as in this instance, there is almost no evidence for the presence of cytochrome oxidase (601 m μ peak in the reduced state) or of flavoprotein (450 m μ peak in the oxidized state). More commonly, however, a small 601 m μ peak is present.

The precipitate from Preparation 0-2 may be further treated with sodium desoxycholate. The precipitate is taken up in 0.1 m Na₂HPO₄-KH₂PO₄ buffer of pH 7.4 and reduced to a uniform suspension in a glass tissue homogenizer. More buffer is added until the final volume is equal to

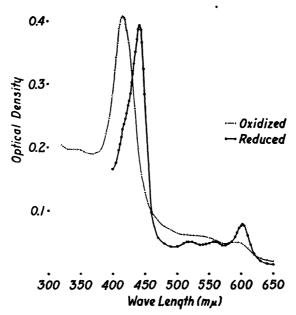


Fig. 3. Preparation 2-3

the volume of the insoluble preparation first taken for Preparation 0-2. To each ml. of the suspension there are added 30 mg. (3 per cent) of sodium desoxycholate in a cold mortar with grinding or in a cold beaker with stirring and the mixture is centrifuged in the cold for 1 hour at 25,000 \times g. The supernatant (Preparation 2-3) can then be lyophilized. The Q_{0_2} protein (25°) values of the dry preparations range from 2080 to 2880. The one described here had a Q_{0_2} protein of 2880. The absorption curves of a solution, made by dissolving 1 ml. equivalent in 5 ml. of cold distilled water is expressed in terms of optical density per mg. of protein (K = D/CL), and indicates the presence principally of cytochrome oxidase (Fig. 3). The curve of the oxidized enzymes has a peak at about 416 m μ (cyto-

chrome oxidase) and another at 590 to 595 m μ (cytochrome oxidase). The curve of the reduced enzymes has a peak at 442 m μ (cytochrome oxidase), a hump with a maximum at about 523 m μ (cytochrome b), another hump with a maximum at 560 m μ (cytochrome b), and a peak at 602 m μ (cytochrome oxidase).

Series Fractionation—It was previously demonstrated (6) that the addition of 1 per cent of sodium desoxycholate to an insoluble preparation would dissolve approximately 25 per cent of the proteins and that the solution would exhibit little or no oxidase activity. This fact and a study of the absorption curves for Preparations 0-2 and 2-3 suggested the successive addition of 1 per cent of desoxycholate to separate the constituent enzymes. An insoluble preparation, precipitated three times by acetate, is used rather than a preparation precipitated only once, because it is assumed that the former is less contaminated by hemoglobin. Each ml. of the insoluble preparation is treated with 10 mg. of sodium desoxycholate in a cold beaker with stirring and then centrifuged in the cold at 25,000 \times g for 1 hour. The supernatant, which is usually somewhat opalescent, is poured off and termed Fraction A. The precipitate is taken up in 0.1 M Na₂HPO₄-KH₂PO₄ and reduced to a uniform suspension in a glass tissue homogenizer. More buffer is added until the volume of the suspension is equal to the volume of the insoluble preparation first To each ml. of the suspension are added 10 mg. of sodium desoxycholate in a cold beaker with stirring. Following centrifugation, as before, the supernatant, termed Fraction B, is poured off and lyophilized. The precipitate is further treated as just described until five fractions have been obtained. The preparation is sometimes complicated by the formation of a gel when either the fourth or the fifth fraction is being stirred in the beaker. Warming the beaker in the hand, accompanied by slow stirring, followed by rapid pipetting into the centrifuge tubes, usually suffices to delay the gel formation. However, if gelation is suspected, it is best to stop the centrifuge at the end of \(\frac{1}{2} \) hour and to inspect the tubes. If a gel layer is present, the tubes should be warmed in the hand, the contents stirred slowly until the gel is dispersed, and promptly placed in the centrifuge.

For spectral analysis Fractions A and B were so prepared that each ml. equivalent was dissolved in 2 ml. of distilled water. Fractions C, D, and E were made so that each ml. equivalent was dissolved in 1 ml. of distilled water. The observed density values were divided by the mg. of protein in the preparation (K = D/CL) and plotted against the wavelength. The curves for Fraction A $(Q_{02}$ protein (25°) 87) are presented in Fig. 4. The curve for the oxidized preparation has a peak at 410 m μ . The curve for the reduced preparation has a peak at 420 m μ , a hump with

a maximum at 548 m μ , and another small peak at 595 m μ . This fraction, which is always opalescent and, therefore, has an uncertain baseline, gives indications of containing small amounts of cytochromes c, b, and oxidase.

Fraction B (Q_{0} , protein 1430) has a peak at 414 m μ (cytochrome b) in the oxidized state (Fig. 5) which is skewed toward 408 m μ (cytochrome c). In the reduced state there is a peak at 428 m μ (cytochrome b) which is unsymmetrical on the left and gives indications of a component at 415 m μ (cytochrome c). The hump with a plateau at 525 to 530 m μ and the peak at 560 m μ which is slightly skewed toward 550 m μ indicate the presence of cytochrome b and of a little cytochrome c. The presence of cytochrome oxidase is indicated by the plateau at 599 to 605 m μ .

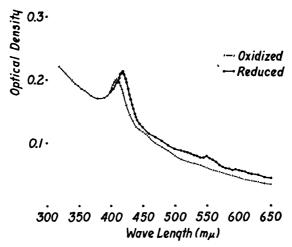


Fig. 4. Fraction A

Fraction C (Q_{02} protein 1840) has a peak on the curve for the oxidized system at 416 m μ (Fig. 6). In the reduced state there is a peak at 428 m μ (cytochrome b), with an accompanying component absorbing strongly at about 440 m μ (cytochrome oxidase). There are peaks at 528 m μ (cytochrome b), 558 m μ (cytochrome b), and 600 m μ (cytochrome oxidase).

Fraction D (Q_{02} protein 650) in the oxidized state (Fig. 7) has a peak at 418 m μ and a small hump with a plateau at 590 to 595 m μ , both of which belong to cytochrome oxidase. The peak at 440 m μ in the reduced state belongs to cytochrome oxidase. It is markedly skewed toward the blue by a component absorbing at about 430 m μ (cytochrome b). The hump with a plateau at 510 to 530 m μ and the 560 m μ peak belong to cytochrome b. The 601 m μ peak belongs to cytochrome oxidase. Frac-

tion E can be shown to have cytochromes b and oxidase, although in lesser amounts. The curves for Fraction E are not given because they

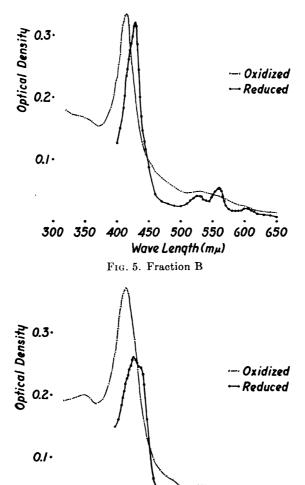


Fig. 6. Fraction C

300 350 400 450 500 550 600 650 Wave Length (mμ)

are very irregular after having been expressed in terms of optical density per mg. of protein (K = D/CL). The protein content is low and all density readings are, therefore, multiplied by a large conversion factor.

A summary of the absorption values obtained following fractionation is given in Table I where the values for cytochrome b and cytochrome

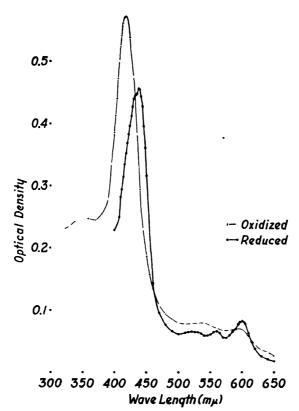


Fig. 7 Fraction D

TABLE I
Absorption Maxima

Preparations		Cytochrome b Cytochro					ne oxidase*		
	Oxidized		Reduced		Ox	idızed	Reduced		
	mμ	mµ	πμ	mμ	mμ'	mμ	mµ.	196 JL	
This paper	414	428	525	560	418	590-595	440	601	
Keilin and Hartree (3)		432	530	564			448	600	

^{*} Keilin and Hartree's cytochrome as.

oxidase are presented. Cytochrome b in the oxidized state has a prominent peak at 414 m μ . In the reduced state it has peaks at 428, 525, and 560 m μ . The relative heights of these three peaks (the 428 m μ peak being

very prominent) and the position of the peaks (compare the values for cytochrome c, 415, 520, and 550 m μ) strongly suggest that cytochrome b is an iron-porphyrin enzyme. Our values for reduced cytochrome b are consistently lower than the values reported by Keilin and Hartree (3) (see Table I). We have, however, repeatedly obtained the same values with several spectrophotometers for either beef or hog heart. Furthermore, we have always checked our instruments against cytochrome c prepared from beef heart and have obtained a characteristic curve for the reduced compound.

Oxidized cytochrome oxidase has a high, sharp peak at 418 m μ and a low, flat peak at 590 to 595 m μ . In the reduced state it absorbs markedly at 440 m μ and at 601 m μ . Our 440 m μ value does not agree with that of 448 m μ given by Keilin and Hartree. The fact that there are only two peaks for cytochrome oxidase in the reduced state and three peaks for cytochrome c and cytochrome b suggests that cytochrome oxidase differs from these compounds.

Copper and Iron Analyses—A large amount of an insoluble heart muscle suspension (approximately 200 ml.), precipitated once by acetate, was made from beef heart by use of buffers that had been prepared with water redistilled from Pyrex glass. The buffer salts were of reagent grade and were found to be practically free of copper and iron. Preparations 0-4, 0-2, and 2-3 and Fractions A, B, C, and D were prepared from this insoluble suspension and lyophilized for use. The sodium desoxycholate was precipitated as desoxycholic acid five times by the addition of $\rm H_2SO_4$ (redissolved each time with NaOH) and finally adjusted to its initial pH and lyophilized to yield a preparation free of copper and very low in iron. All glassware was carefully washed with water that had been redistilled from Pyrex glass. The values obtained for protein, oxygen uptake, copper, and iron, and for the height of the 601 m μ peak on the absorption curve of the reduced preparation are given in Table II. A discussion of the values in each column follows.

Protein—4 per cent of desoxycholate dissolves about 80 per cent of the proteins contained in the insoluble preparation. The sum of the proteins dissolved by 2 and 3 per cent of desoxycholate added successively (Preparation 0-2 \pm 2-3) is about 95 per cent of the proteins contained in the insoluble preparation.

15 Minute Oxygen Uptake—In the test for activity all the enzyme solutions were used in the dilute (1:25) form. 0.25 ml. of Preparations 0-4 and 2-3, 0.5 ml. of the insoluble preparation and Preparation 0-2, and 0.75 ml. of Fractions A through D were used.

1 Hour Oxygen Uptake—This is a calculated value. Column 2 is multiplied by 4 to increase the time to 1 hour, and multiplied by 25 to account for the dilution.

TABLE II
Angluses of Various Prenarations

	Oxygen uptake-601 mµ ratio (3) ÷ (11)	(14)	c.mm. per density wnit				24,400		42,700	18,500	4,800
	Iron-601 m# ratio (9) + (11)	(13)	y per density unit				5.2		15.9	2.2	<1.4
	Copper- 601 mµ ratio (6) + (1)	(12)	y per density unit				4.8		7.1	6.3	6.2
	Height of 601 mµ peak*	(11)	density		1.180‡	\$016.0	0.460	0.340‡	0.082	9 .092	0.147
	Iron- protein ratio (9) + (1)	(10)	y per mg.	0.54	0.49	0.41	0.47	99.0	0.55	0.20	<0.19
ons	Iron per ml	6	٨	12.1	8.8	6.7	2.4	3.8	1.3	0.2	<0.2
reparan	Iron in sample analyzed	(8)	٠	12.1	8.8	20.1	7.3	15.3	4.0	6.0	>0.6
analyses of various Freparations	Copper- protein ratio. (6) ÷ (1)	3	7 per ms.	0.22	0 17	0.07	0.	0.10	0.24	0.59	0.84
yses of	Copper per ml.*	9	٨	4.9	3.0	1.1	2.2	0 56	0.58	0.58	0.91
mur	Copper in sample analyzed	(2)	7	29 6	15.0	5.4	11.0	5.6	5.8	5.8	9.1
	O_{O_2} protein (25°) (3) + (1)	€	c.mm. per mg. per hr.	260	1290	20	2220	120	1470	1740	650
	1 hr. oxygen uptake*	(3)	C.MM.	5,800	23,200	1,200	11,200	200	3,500	1,700	200
	15 min. oxygen uptake†	3	. # #.	88	232	12	112	~	35	17	~
	Protein*	Ξ	.su	22.45	17.95	16.25	5.05	5.78	2.38	0.98	1.08
	Preparation No.			Insoluble	0-4	0-5	2-3	¥	m	Ö	D

* Per ml. of concentrated supernatant. † Per ml. of dilute supernatant (1:25). ‡ Value doubtful; see the text for explanation.

 Q_{02} Protein (25°) —These values are fairly typical. The low value for Fraction D, which contains cytochrome oxidase on the basis of its absorption spectra, is difficult to explain. The discrepancy may be due to at least one of the following: (a) an inhibition by desoxycholate of the oxidation of reduced cytochrome c by oxidase in the test for activity (observed both manometrically and spectrophotometrically); (b) denaturation of the cytochrome oxidase during the preparation; (c) a separation of factors not yet recognized.

Copper in Sample Analyzed—5 ml. or 5 ml. equivalents were used in analyzing the insoluble preparation and Preparations 0-4, 0-2, and 2-3. 10 ml. equivalents of Fractions A through D were used. The results are considered to be correct to within ± 10 per cent. The copper contained in Preparations 0-4, 0-2, and 2-3 is non-dialyzable. Samples were dialyzed with slow stirring against 6 liters of distilled water at 4° for 4 days. The copper content and the ratio of copper to protein in each sample was unchanged.

Copper Per Ml.—It is to be noted that the copper content of Fraction D is the highest of all four fractions and that Fraction D always exhibits the most prominent 601 m μ peak.

Copper-Protein Ratio—The value for Preparation 0-2 is low and the value for Preparation 2-3 is high when compared to the insoluble preparation. It is to be noted that Preparation 2-3 had a prominent peak at 601 m μ , while Preparation 0-2 had none. See Figs. 2 and 3 for curves that are almost exact replicas of the curves for these preparations.

Iron in Sample Analyzed—1 ml., and 1 ml. equivalent of the lyophilized preparation, respectively, were used in analyzing the insoluble preparation and Preparation 0-4. 3 ml. equivalents were taken of Preparations 0-2 and 2-3 and of Fractions B and D, and 4 ml. equivalents of Fractions A and C. The results are considered to be correct to within ± 10 per cent. The iron contained in Preparations 0-4, 0-2, and 2-3 is non-dialyzable. Samples were dialyzed with slow stirring against 6 liters of distilled water at 4° for 4 days. The iron content and the iron-protein ratios were unchanged.

Iron Per Ml.—It is to be noted that the iron content decreases markedly in Fractions A to D. The value for Fraction D is essentially zero.

Iron-Protein Ratio—The ratios are essentially constant for all preparations except that Fraction C has a low ratio and Fraction D has a ratio somewhere near zero.

Height of 601 mµ Peak—These density values were obtained with the spectrophotometer. The values for Preparations 0-4 and 0-2 and for Fraction A are not considered significant because the opalescence (probably due to fat) raised the base-line to an unknown height above zero.

Ratio of Copper to 601 $m\mu$ Peak—The ratios are fairly constant. The correlation is considered good for Preparation 2-3 and for Fractions B, C, and D. These constant ratios are considered to indicate that copper is associated with the cytochrome component that absorbs light at 601 $m\mu$.

Ratio of Iron to 601 $m\mu$ Peak—These ratios are not constant. They range from 15.9 for Fraction B to near zero for Fraction D. The poor correlation is interpreted to mean that iron is not contained in the component that absorbs at 601 $m\mu$.

Ratio of Oxygen Uptake to 601 m μ Peak—The ratios are not constant, ranging from 4800 to 42,700. Unless the ratios are inaccurate for the reasons presented above under " Q_{O_2} protein (25°)," this would indicate that the oxygen uptake is not proportional to the concentration of the component which absorbs at 601 m μ . However, the preparations that have a prominent 601 m μ peak, with the exception of Fraction D (see Figs. 1, 3, 5, and 6), also have a high Q_{O_2} protein.

DISCUSSION

A comparison of the absorption spectra of our Preparation 0-4 and of Straub's preparation (2), which he made with sodium cholate, ammonium sulfate, sodium hydrosulfite, and heat, reveals a marked similarity. However, Straub reports that another of his preparations, which is essentially free of cytochrome b and which has an absorption curve very much like our Preparation 2-3, has a $Q_{\rm O_2}$ protein of 300 at 38°. This is to be contrasted to the $Q_{\rm O_2}$ protein of 2880 at 25° for our Preparation 2-3.

Keilin and Hartree (3) have reported the presence of two cytochrome a components, namely, cytochromes a and a₃. According to these authors the two enzymes have the same absorption spectra and can be differentiated best by forming the carbon monoxide compound of the reduced cytochrome a_3 . Straub arrives at the same conclusion, although the peak that he attributes to cytochrome a is more probably the 428 m μ peak of cytochrome b. We have adopted the term oxidase for the compound which absorbs strongly at 442 and 601 m μ , and we have compared its absorption peaks with Keilin and Hartree's cytochrome a₃. Keilin and Hartree suggest that if cytochrome a_3 has a third band in the visible region it is masked in their preparations by the 550 and 560 m μ bands of the cytochromes c and b. We have never encountered a third peak for cytochrome oxidase. enzyme, which absorbs at 442 m μ and at 601 m μ , appears to be the one that oxidizes hydroquinone in the presence of cytochrome c, oxidizes reduced cytochrome c, and is inhibited by carbon monoxide, cyanide, and azide (6). Although the correlation between the oxygen uptake catalyzed by a preparation and the height of the peak at 601 mu is not always good, in general those preparations that have a prominent peak at 601 m μ also have a high Q_0 , protein. The poor correlation may be due in part to the fact that sodium desoxycholate interferes with the oxidation of reduced cytochrome c by cytochrome oxidase in the test of activity.

There is a marked difference in color between Preparations 0-2 and 2-3. A solution of Preparation 0-2, although somewhat opalescent, is red in color and highly suggestive of a solution of cytochrome c. In view of the spectrophotometric observation that this preparation contains cytochrome b with but small amounts of cytochrome c, it appears likely that cytochrome b is an iron-porphyrin compound. In marked contrast to the color of Preparation 0-2 is the yellow-green of Preparation 2-3, especially when it can be demonstrated spectrophotometrically that the latter contains very little cytochrome b. Since many copper compounds are green in color, the suggestion that cytochrome oxidase is a copper-containing enzyme is strengthened.

Both Keilin and Hartree (3) and Graubard (8) have suggested that cytochrome oxidase might be a copper-containing enzyme. The conclusion of Keilin and Hartree is based on such observations as the universal distribution of intracellular copper, the facility with which copper salts oxidize reduced cytochrome c, the work of Cohen and Elvehiem discussed below, and a certain similarity in properties between cytochrome oxidase and polyphenol oxidase, which is a copper-protein compound. Graubard, more directly, inhibited the oxidation by cytochrome oxidase of p-phenylenediamine by adding certain reagents that are known to combine with copper. His experiments were in accord with the earlier experiments of Cohen and Elvehjem (15) and Schultze (16, 17). Cohen and Elvehjem found a decreased oxidase activity in the livers of rats made anemic on a milk diet. The feeding of copper increased the oxidase activity, while the feeding of iron did not. Schultze found the oxidase activity of rat liver, heart, and bone marrow to be greatly reduced on a copper-deficient diet. He as well as Cohen and Elvehjem concluded that copper was essential for the formation and maintenance of cytochrome oxidase activity. Our experiments offer direct evidence from analyses and indirect evidence from comparison with the absorption spectrum of reduced cytochrome c that cytochrome oxidase is a copper-containing enzyme and not an iron-containing enzyme.

SUMMARY

- 1. Cytochrome oxidase and cytochrome b of mammalian heart muscle have been almost completely separated by the use of sodium desoxycholate.
- 2. Cytochrome oxidase in the oxidized state (with sodium desoxycholate present) has a high, sharp peak in its absorption curve at 418 m μ and a very low, flat peak at 590 to 595 m μ . In the reduced state it absorbs

strongly at 440 and 601 m μ . The absence of a third peak, the presence of which is so characteristic of cytochrome c, suggests that cytochrome oxidase differs in its composition from this compound.

- 3. Cytochrome b in the oxidized state (with sodium desoxycholate present) displays a prominent absorption peak at 414 m μ . In the reduced state it absorbs strongly at 428, 525, and 560 m μ . A consideration of the general contour of the curves, the relative heights of the peaks, and the position of the peaks when compared with the same for cytochrome c suggests that cytochrome b may be an iron-porphyrin enzyme.
- 4. The good correlation obtained with several fractions between the copper content and the height of the 601 m μ peak in the reduced curve is considered to indicate that cytochrome oxidase is a copper-containing enzyme. This conclusion is strengthened by the observation that the correlation between the iron content and the height of the 601 m μ peak is poor.
- 5. The lack of correlation in many instances between the oxygen uptake catalyzed by a preparation and the absolute height of the absorption peak at 601 m μ remains unexplained. In general, however, the preparations that have a prominent 601 m μ peak in the reduced state also have a high Q_{0z} protein.

The authors wish to thank Dr. Albert E. Sobel and Dr. Samuel Natelson of the Division of Biochemistry and the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, for most generously making available the facilities of their laboratories during the early part of this investigation.

BIBLIOGRAPHY

- 1. von Euler, H., and Hellström, H., Z. physiol. Chem., 260, 163 (1939).
- 2. Straub, F. B., Z. physiol. Chem., 268, 227 (1941).
- 3. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 127, 167 (1939).
- 4. Yakushiji, E., and Okunuki, K., Proc. Imp. Acad., Tokyo, 17, 38 (1941).
- Wainio, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., Science, 106, 471 (1947).
- Wainio, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., J. Biol. Chem., 173, 145 (1948).
- 7. Eichel, B., Cooperstein, S. J., and Wainio, W. W., Biol. Bull., 95, 239 (1948).
- 8. Graubard, M., Am. J. Physiol., 131, 584 (1941).
- 9. Eichel, B., and Wainio, W. W., J. Biol. Chem., 175, 155 (1948).
- 10. Haas, E., J. Biol. Chem., 148, 481 (1943).
- 11. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 125, 171 (1938).
- 12. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., J. Biol. Chem., 124, 733 (1938).
- 13. McFarlane, W. D., Biochem. J., 26, 1022 (1932).
- 14. Lintzel, W., Z. ges. exp. Med., 86, 269 (1933).
- 15. Cohen, E., and Elvehjem, C. A., J. Biol. Chem., 107, 97 (1934).
- 16. Schultze, M. O., J. Biol. Chem., 129, 729 (1939).
- 17. Schultze, M. O., J. Biol. Chem., 138, 219 (1941).

PYRIMIDINE NUCLEOSIDES AS PRECURSORS OF PYRIMIDINES IN POLYNUCLEOTIDES

By EINAR HAMMARSTEN AND PETER REICHARD

(From the Biochemical Department, Karolinska Institutet, Stockholm, Sweden)

AND E. SALUSTE

(From the Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden)

(Received for publication, September 20, 1949)

The purines and pyrimidines, so far the only known nitrogen-containing components of the polynucleotides, differ, with the exception of adenine, from most other nitrogenous substances, e.g. amino acids, in that when administered with the food they are not utilized by the rat. This was first shown by Plentl and Schoenheimer (1), who fed N¹⁵-labeled guanine, uracil, and thymine to rats and isolated the bases from the polynucleotides. They showed that in no case had isotope entered the purines or pyrimidines. The only substances containing the isotope in a significant amount were allantoin, after feeding guanine, and urea and ammonia after the pyrimidines were fed. These products were isolated from urine. The same negative results were obtained by Bendich, Getler, and Brown (2) with cytosine as the isotopic precursor.

From these experiments the conclusion might be drawn that the pyrimidines present in food in the form of polynucleotides cannot act as precursors for the synthesis of new polynucleotides by the rat. This does not necessarily follow if, as is generally assumed, the digestive enzymes do not split off the free bases from dietary polynucleotides. Nucleosides formed by partial hydrolysis may be absorbed through the intestinal wall and these, unlike the free pyrimidines, might then be utilized for the synthesis of new polynucleotides by the animal.

That a difference exists between free pyrimidines and pyrimidine nucleosides or nucleotides for certain organisms has been shown by Loring and Pierce (3) in experiments on mutants of *Neurospora* which require uracil for growth. Cytosine did not permit any growth. Both uridine and cytidine or the corresponding nucleotides could, however, be used instead of uracil. Furthermore the nucleosides and nucleotides allowed 10 to 60 times more growth than did an equimolar amount of uracil. The much higher activity of the nucleosides compared with that of the free bases may indicate a fundamental difference between the metabolism of free pyrimidine and pyrimidine bound to ribose. This difference may be valid for the rat also.

In order to investigate this question, cytidine and uridine, labeled in the rings and the amino group with N¹⁵, have been injected into rats. A preliminary report of these experiments has been published previously (4). At about the same time Brown *et al.* (5) reported the results of experiments in which a biologically (N¹⁵) marked hydrolysate of yeast nucleic acid was administered to rats. The isolated mixed pyrimidines contained a significant amount of the isotope, showing that the hydrolysate served as a precursor for the pyrimidines in polynucleotides.

In the present investigation the N¹⁵ nucleosides were prepared by growing yeast on a medium containing ammonia with an excess of N¹⁵ as the

Table I
Injection of Cytidine-N¹⁵ into Rats (Total of 25 Mg. per 100 Gm. of
Body Weight during 3 Days)

For the value E_{max} /(microgram of N per ml.) see Reichard (7, 14) and Edman et al. (9).

		N ¹⁵ atom per cent excess	$\frac{E_{\text{max.}}}{\gamma \text{ N per ml.}}$	Per cent N ¹⁵ calculated on basis of 100 per cent N ¹⁵ in nucleoside injected
	Injected, cytidine	6.93	0.297	100
Isolated	PNA		TO A SECURITION OF THE PARTY.	N. COMM.
	Cytidine	0.463	0.303	7.20
	Uridine	0.385	0.321	5.94
	Adenine	0.003	0.178	0.05
	Guanine	0.005	0.162	0.08
	DNA			
	Cytosine	0.281	0.228	4.37
	Thymine	0.101	0.275	1.57
	Adenine	0.074	0.169	1.15
	Guanine	0.028	0.167	0.44
	"Protein"	0.006		0.10

only source of nitrogen. Pyrimidine nucleosides were isolated from the ribose nucleic acid (PNA) of this yeast according to methods worked out in this laboratory (6-8).

Two rats each received subcutaneously a total of 25 mg. of one of the nucleosides per 100 gm. of body weight, divided into six injections at twelve hourly intervals. The rats were killed 12 hours after the last injection and the polynucleotides were isolated from the pooled internal organs. The PNA and the desoxyribose nucleic acid (DNA) were isolated (6) and the bases and nucleosides, respectively, were prepared from each fraction (7–9, 14) and analyzed for N¹⁵.

The results from the cytidine injection (Table I) clearly indicate that

this nucleoside was a good precursor not only for the pyrimidines of PNA but also for those of DNA. As might be expected, the cytosine in both cases had a higher isotope content than the other pyrimidine. The purines from PNA did not contain a significant amount of N¹⁵ and the same was true of the trichloroacetic acid-insoluble or "protein" fraction. In the case of the DNA the purines, especially adenine, contained a significant amount of the isotope.

Table II
Injection of Uridine-N¹⁵ into Rats (Total of 25 Mg. per 100 Gm.
of Body Weight during 3 Days)

		N ¹⁵ atom per cent excess	$\frac{E_{\max}}{\gamma \text{ N per ml.}}$	Per cent N ¹⁵ calculated on basis of 100 per cent N ¹⁵ in nucleoside injected
	Injected, uridine	11.20	0.325	100
Isolated	PNA			
	Cytidine	0.053	0.298	0.47
	Uridine	0.047	0.325	0.42
	Adenine	0.010	0.179	0.09
	Guanine	0.009	0.166	0.08
	DNA			
	Cytosine	0.055	0.218	0.49
	Thymine	0.039	0.269	0.34
	Adenine	0.033	0.181	0.29
	Guanine	0.049	0.159	0.44
	"Protein"	0.012		0.11

When *uridine-N*¹⁵ was injected, the isotope entered the different nitrogen-containing substances of both PNA and DNA in rather small amounts (Table II), the pyrimidines showing only a slightly higher isotope content than the purines.

DISCUSSION

Cytidine As Precursor—The present results show that cytidine, administered in small amounts, can be utilized by the rat for the synthesis of new polynucleotides. The isotope in the cytosine of the PNA is so little diluted compared with the administered cytidine that the isotope content in the pyrimidines of the polynucleotides cannot be due to the isotopic amino group of the administered cytidine. This is especially true if one considers the low isotope content in purines from PNA and proteins. It is obvious that the pyrimidine ring of cytidine must have entered the different pyrimidines in the PNA and DNA.

A very interesting finding is the rather high amount of isotope in the

pyrimidines of the DNA. The turnover ratio PNA:DNA is about 2:1 for cytosine, a finding which agrees well with the results obtained by Hammarsten and Hevesy (10) for the phosphorus of the whole rat and is in marked contrast to the results obtained by Brown et al. (11) with adenine-N¹⁵ as precursor for purines from polynucleotides (29:1).

The occurrence of the isotope in the DNA pyrimidines suggests that the rat is able to form desoxyribose from ribose when the sugar is bound to cytosine. This agrees with the conclusion of Bendich, Getler, and Brown (2) that free cytosine cannot be utilized for the synthesis of DNA pyrimidines. Thus cytosine cannot be split off from ribose and then linked to desoxyribose for the synthesis of the DNA. This is in agreement with the results obtained by Bredereck et al. (12) concerning the point of attachment of desoxyribose to the pyrimidines. These authors found by methylation experiments that the sugar was attached in position 3 of cytosine and thymine. According to our hypothesis, the attachment of desoxyribose to the pyrimidines in DNA is the same as that of the ribose to the pyrimidines in PNA. The latter has been firmly established to be in position 3.

The relatively high content of the isotope in the purines of the DNA, especially in adenine, was unexpected. This cannot very well be ascribed to the amino group of the administered N¹⁵-cytidine because the purines from the PNA contain much less of the isotope. The data may possibly indicate some connection between the turnover of purines of the DNA and pyrimidines. This point, however, needs further confirmation.

Uridine As Precursor—When the same amount of uridine-N¹⁵ was injected into the rat, the results were quite different from those obtained with cytidine-N¹⁵. The dilution of the isotope in the PNA pyrimidines was about 15 times higher than when cytidine was used. Furthermore, the isotope, especially in the case of DNA, was distributed much more evenly between the pyrimidines and the purines. It would seem therefore that the utilization of uridine for the synthesis of polynucleotide pyrimidines is very low and is not very specific.

The present experiments also give some evidence concerning the reactions involved in the utilization of orotic acid for the synthesis of the pyrimidines. It has been shown that orotic acid may act as a precursor for pyrimidines in both PNA and DNA (13, 14), but it is not clear how this compound is transformed into uridine and cytidine. Previous experiments on Neurospora (3) indicated that the first step might involve a transformation of the orotic acid riboside to uridine. This was disproved, however, by experiments in which the distribution of the isotope between uridine and deaminated cytidine (14) led to the conclusion that uridine and cytidine were formed from orotic acid independently of each other. The present findings and the previous experimental data suggest that

orotic acid after amination is first transformed to cytidine and that uridine is then formed from this cytidine.

On the other hand, this general concept of the formation of uridine via cytidine is contradictory to the results of studies of the turnover of PNA pyrimidines in rats after the injection of glycine-N¹⁵ (14). In these experiments in which the ribosides were prepared from the PNA of different organs, the turnover of the cytosine ring was generally lower than that of uridine. In this case uridine did not appear to have been formed from cytidine.

A similar situation has been found previously in respect to the synthesis of polynucleotide guanine from adenine. This biosynthesis has been proved by the experiments of Brown et al. (15) with adenine-N¹⁵ as precursor. When glycine-N¹⁶ was used as precursor for polynucleotide purines, however, this synthetic route was not found in all the organs investigated (14). It is concluded that one synthetic route does not exist for pyrimidines and purines, but that these substances may be built up in different ways and from different precursors.

SUMMARY

Biologically marked cytidine and uridine have been isolated from yeast grown on a medium containing ammonia with an excess of N¹⁵ as the source of nitrogen.

The results of the separate injection of the nucleosides into rats show that cytidine is a precursor of PNA and DNA pyrimidines.

BIBLIOGRAPHY

- 1. Plentl, A. A., and Schoenheimer, R., J. Biol. Chem., 153, 203 (1944).
- 2. Bendich, A., Getler, H., and Brown, G. B., J. Biol. Chem., 177, 565 (1949).
- 3. Loring, H. S., and Pierce, J. G., J. Biol. Chem., 153, 61 (1944).
- 4. Hammarsten, E., Reichard, P., and Saluste, E., Acta chem. Scand., 3, 433 (1949).
- Roll, P. M., Brown, G. B., Di Carlo, F. J., and Schultz, A. S., J. Biol. Chem., 180, 333 (1949).
- 6. Hammarsten, E., Acta med. Scand., suppl. 196, 634 (1947).
- 7. Reichard, P., Nature, 162, 662 (1948).
- 8. Reichard, P., J. Biol. Chem., 179, 763 (1949).
- Edman, P., Hammarsten, E., Löw, B., and Reichard, P., J. Biol. Chem., 178, 395 (1949).
- 10. Hammarsten, E., and Hevesy, G., Acta phys. Scand., 11, 335 (1946).
- Brown, G. B., Petermann, M. L., and Furst, S. S., J. Biol. Chem., 174, 1043 (1948).
- 12. Bredereck, H., Müller, G., and Berger, E., Ber. chem. Ges., 73, 1058 (1940).
- Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., von Ubisch, H., and Bergström, S., J. Biol. Chem., 179, 169 (1949).
- 14. Reichard, P., Acta chem. Scand., 3, 422 (1949).
- Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., J. Biol. Chem., 172, 469 (1948).

THE INHIBITION OF THE SUCCINOXIDASE SYSTEM BY α-TOCOPHERYL PHOSPHATE AND SODIUM DODECYL SULFATE*

By M. RABINOVITZ AND PAUL D. BOYER

(From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul)

(Received for publication, October 3, 1949)

In attempts to gain information on the biological function of α -tocopherol, a number of investigations have been made dealing with the inhibitory effect of α -tocopheryl phosphate (α -TPh) on enzyme systems. The results obtained with the phosphate ester have served as a basis for speculations on the biological function of vitamin E and have led to the suggestion that the vitamin functions to control the rate of biological oxidations (1), to prevent the breakdown of diphosphopyridine nucleotide (DPN) (2), and to control intravascular blood clotting (3). The principal studies have been concerned with the inhibition of the succinoxidase system by α -TPh. These and other related studies have been recently briefly reviewed (4). In addition, α -TPh has been shown to inhibit trypsin (3), liver acid phosphatase (5), lactic dehydrogenase (2), and DPN nucleosidase (6).

The experiments reported herein were undertaken to ascertain whether the varied effects of α -TPh might be related to the properties of tocopheryl phosphate as an anion with a large non-polar group. Such anions are known to act as protein denaturants and enzyme inhibitors (7). In addition, experiments are reported which give a further understanding of the mechanism by which dodecyl sulfate and α -TPh inhibit the succinoxidase system.

Methods

The manometric succinoxidase¹ assay procedure employed was essentially that of Schneider and Potter (8) for rat liver homogenates with the use of twice recrystallized sodium succinate as substrate. Solutions of the inhibitors, the disodium salt of dl- α -tocopheryl phosphate² (α -TPh) or sodium dodecyl sulfate³ (SDS), were added at the expense of water. The

- * Paper No. 2476, Scientific Journal Series, Minnesota Agricultural Experiment Station. Aided by a grant from the Nutrition Foundation, Inc., New York.
- ¹ The term succinoxidase refers to the complete system, including, besides succinic dehydrogenase, the cytochrome components enabling reaction with molecular oxygen.
 - ² Kindly supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey.
- ³ A purified preparation kindly supplied by E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

cytochrome c was prepared according to Potter's modification (9) of the method of Keilin and Hartree (10). Trials with different levels of cytochrome c were made to insure that cytochrome was not the limiting component.

In the cytochrome oxidase studies two tissue concentrations were used. The higher level of tissue was the same as in dehydrogenase studies with methylene blue and was used to make the observations more directly comparable as to the amount of tissue and inhibitor used. Since the amount of cytochrome c was not sufficient to saturate the cytochrome oxidase system, other experiments were performed with a lower tissue concentration in which the cytochrome c concentration was sufficient for saturation. The addition of Ca⁺⁺ and Al⁺⁺⁺ as activators (8) was not made in the studies with heart homogenate because the cations form insoluble salts with α -TPh. These cations have not been proved to be essential for the activity of the succinoxidase system. The activation seems to be related to the colloidal phosphates which these cations form with the phosphate buffer (11).

For the cytochrome oxidase assay, ascorbate was used to maintain the cytochrome c in the reduced form, as has been done by previous investigators (8). The flasks with the higher tissue level contained 0.50 ml. of 0.21 m phosphate buffer, pH 7.4, 0.50 ml. of 0.50 m sodium succinate, 0.80 ml. of 1.8×10^{-4} m cytochrome c, 0.50 ml. of 5 per cent rat heart homogenate, and sufficient water to make a total volume of 3.4 ml. The flasks with lower tissue level contained 0.50 ml. of phosphate buffer, 0.30 ml. of sodium succinate, and 1.3 ml. of cytochrome c, of the same corresponding concentrations as for the higher tissue level, together with 0.5 ml. of a 1 per cent rat heart homogenate and water to make a total volume of 3.4 ml. The side arms in all cases contained 0.4 ml. of 0.11 m sodium ascorbate, pH 7.0, which had been prepared immediately before using.

Readings with succinate as the only substrate were taken during the first 10 minute interval. The values represented the succinoxidase activity. The side arms containing the ascorbate were then tipped and the oxygen uptake between the 20 to 30 minute interval represented the cytochrome oxidase activity. Correction for autoxidation of ascorbate was made as suggested by Schneider and Potter (8).

In the study of the succinic dehydrogenase by the methylene blue technique the following additions were made to the flasks: 0.50 ml. of 0.21 m phosphate buffer, pH 7.4, 0.50 ml. of 0.5 m sodium succinate, redistilled water to make 3.4 ml. final total volume, 0.1 ml. of 0.15 m potassium cyanide (when used), 0.4 ml. of either 0.01 m or 0.05 m methylene blue (side arm), the α -TPh or SDS solution, and 0.5 ml. of 5 per cent rat heart homogenate.

In all experiments with inhibitors, controls were run simultaneously with the same enzyme preparations because variations were noted in the enzymatic activity of different tissue preparations.

EXPERIMENTAL AND DISCUSSION

Inhibition of Succinoxidase System—The behavior of α -TPh was compared with that of surface-active agents, in particular with dodecyl sulfate. Both α -TPh and SDS inhibited the succinoxidase system of rat liver homogenates at similar low concentrations. Trials at different levels

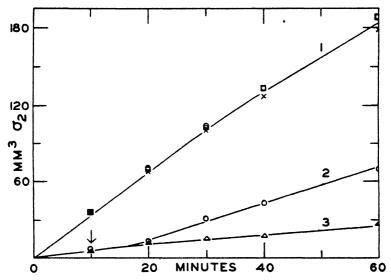


Fig. 1. The effect of heat-inactivated liver homogenate upon the α -TPh inhibition of succinoxidase. Curve 1, \bigcirc , succinoxidase assay as described in the text; \times , assay with 0.40 ml. of heated 10 per cent liver homogenate; \square , assay in the presence of 1.9×10^{-4} m α -TPh to which 0.40 ml. of 10 per cent heated liver homogenate had been added before the active homogenate. Curve 2, as in \square above, but with the heated homogenate added after measuring the oxygen uptake for 10 minutes, as indicated by the arrow. Curve 3, inhibition of succinoxidase by 1.9×10^{-4} α -TPh.

showed that concentrations of 1.9 and 2.8×10^{-4} m respectively were just sufficient for complete inhibition, while a concentration of 5.0×10^{-5} m of either inhibitor produced only 5 to 10 per cent inhibition. In addition, low concentrations of commercial anionic, cationic, and non-ionic detergents and of fatty acid soaps were found to inhibit succinoxidase. This is in harmony with a recent report of Hockenhull (12).

A further similarity of the actions of α -TPh and SDS was evident from comparisons of the effect of other proteins on the inhibition produced by

these substances. The mixing of α -TPh and of SDS with bovine serum albumin⁴ before addition to the flasks decreased their inhibitory effects. Levels of 5 to 7 mg. of albumin per flask decreased the inhibition of α -TPh by about 50 per cent, and that of SDS by about 90 per cent. Serum albumin, in common with a number of proteins, is known to combine readily with anions having large non-polar groups (7, 13). Also, as shown in Fig. 1, the addition of heat-inactivated homogenate to flasks containing sufficient α -TPh otherwise to cause considerable inhibition protected the system completely. Closely analogous results were obtained for SDS inhibition. These observations are best explained by assuming a combination of the α -TPh or SDS with components of the inactivated homogenate, thereby protecting the succinoxidase of the active homogenate added subsequently.

Effect of α -TPh and SDS on Cytochrome c and Cytochrome Oxidase—Since cytochrome c and cytochrome oxidase are components of succinoxidase, the inhibition of the system by α -TPh may be a reflection of its effect upon one of these carriers. It has been reported by Jacobi et al. (5) that the inhibition may largely be due to an effect on cytochrome c, while Houchin (14) found that α -TPh inhibits the reduction of ferricytochrome c by ascorbate. Also, in a short discussion of the properties of cytochrome c, Keilin and Hartree (15) reported modification of its absorption bands by SDS, and Sevag and Ross (16) found a disappearance of specific cytochrome bands in bacteria upon treatment with zephiran, a cationic detergent.

When either α -TPh or SDS was added to a solution of cytochrome c in phosphate buffer, a marked decrease in the characteristic absorption peaks of ferrocytochrome c occurred, as shown in Fig. 2. In these experiments, both the cytochrome c and the phosphate buffer were in the same concentrations as those used in the manometric experiments with liver homogenate. The effect occurred when the α -TPh or SDS was added either before or after the reduction of the cytochrome. No interference in the reduction was apparent. Concentrations of α -TPh slightly above those required to inhibit the succinoxidase system caused definite decreases in the absorption peaks, but relatively higher concentrations of SDS were needed. Higher concentrations of α -TPh or SDS were effective in reducing the maxima of the absorption bands still more. Although these compounds produced a marked change in the spectrum of ferrocytochrome c, a corresponding effect on the oxidized form was not found. Bovine serum albumin, when present as a 2 per cent solution with either the reduced or oxidized cytochrome c before the addition of α -TPh or SDS, completely prevented the spectral changes.

⁴ Kindly supplied by Armour and Company, Chicago, Illinois.

In view of the rather striking effect of α -TPh on the absorption spectrum of cytochrome c, manometric studies were undertaken to ascertain

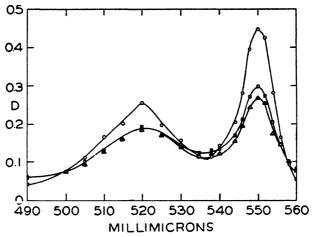


Fig. 2. The effect of α -TPh and SDS upon the absorption spectrum of cytochrome c. To test-tubes were added 0.5 ml. of 0.21 m phosphate buffer, pH 7.4, 0.5 ml. of 0.96 \times 10⁻⁴ m oxidized cytochrome c, α -TPh or SDS solutions, and water to make a total volume of 2.8 ml. After incubation at 38° for 15 minutes, 0.2 ml. of a solution of sodium hydrosulfite in phosphate buffer was added to each tube and the absorption spectrum measured with a Beckman spectrophotometer. \bigcirc , without α -TPh or SDS, \square , in presence of 9.6 \times 10⁻⁴ m SDS, \triangle , in presence of 2.5 \times 10⁻⁴ m α -TPh.

TABLE I

Effect of Additional Cytochrome c, Included after 10 Minutes, upon Succinoxidase Inhibition by α -TPh and SDS

In all cases the initial amount of cytochrome c was sufficient to saturate the uninhibited system. With SDS inhibition, although some reactivation was obtained after cytochrome addition, the increase attainable did not approach the value of the uninhibited system.

Succinovidase source	amount of cytochrome c		nount of Inhibitor		Oxygen uptake		
Succinomical source			c added after 10 min. α-TPh		0-10 min.	20-30 min.	
	micromole	micromole	₩ × 10 ⁴	w × 10⁴	c.mm.	c.mm.	
Rat liver	0.048	0	0	0	37	36	
44 44	0.048	0.048	1.35	0	6.8	6.8	
" heart	0.090	0	0	0	103	83	
" "	0.090	0.090	1.42	0	32	29	
" "	0.090	0.090	0	2.84	13	28	

whether this effect might offer an explanation for the inhibition of succinoxidase. However, as shown in Table I, when a fresh supply of cytochrome c was added from the side arm to a system partially inhibited by α -TPh, no reactivation was obtained. Some reactivation would have been anticipated if cytochrome c were made the limiting component. The initial presence of twice the usual amount of cytochrome c also did not alter the extent of inhibition. In analogous experiments with SDS, the addition of extra cytochrome c only partially reactivated the system. From these experiments it is apparent that the inhibition of the complete succinoxidase system by α -TPh is not due to action on the cytochrome c component.

Measurements of the effect of α -TPh on rat heart cytochrome oxidase showed that this enzyme was still active in flasks where the succinoxidase system had been completely inhibited by α -TPh. This was ascertained

Table II Effect of α -TPh and SDS on Rat Heart Cytochrome Oxidase For experimental details see the text.

Initial homogenate	Inhibitor concentration	Oxygen uptake			
concentration	Innibitor (oncentration	Succinoxidase	Cytochrome oxidas		
per cent	M × 104	c.mm. O2	c mm. O2		
5	0	131	164		
5	4.75 α-TPh	0	55		
5	4.75 SDS	0	30		
1	0	35	75		
1	0.36 α-TPh	18	37		
1	0.71 "	8	34		
1	1.90 "	0	31		

by the addition of ascorbate from the side arm to such a system, thereby affording a reduction of the cytochrome which was independent of the action of the succinic dehydrogenase. As shown in Table II, at levels of α -TPh or SDS that would completely inhibit the succinoxidase system, the cytochrome oxidase was only partially inhibited. The smaller comparative effect of these inhibitors on the cytochrome oxidase demonstrates that their principal inhibitory action on the complete succinoxidase system is not through an effect on the cytochrome system. It must, therefore, be due either to inhibition of the dehydrogenase or to interference with the interaction of the dehydrogenase and cytochrome systems.

Effect of α -TPh and SDS on Succinic Dehydrogenase—The dehydrogenase component or components of succinoxidase may be studied independently of the cytochrome system by use of methylene blue as a hydrogen acceptor. The use of methylene blue in the presence of cyanide to block cytochrome oxidase gave satisfactory oxygen uptakes with heart but not with liver homogenates; hence heart preparations were used in these studies.

The data presented in Figs. 3 and 4 show that methylene blue additions partially restored the oxygen uptake of preparations completely inhibited by α -TPh or SDS. The change from a complete to a partial inhibition was also obtained when potassium cyanide was present in the medium in addition to α -TPh or SDS.

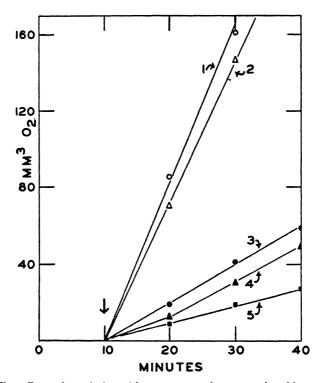


Fig. 3. The effect of methylene blue upon rat heart succinoxidase completely inhibited by α -TPh. Curve 1, methylene blue 6.3×10^{-3} m, potassium cyanide 4.7 $\times 10^{-3}$ m; Curve 2, methylene blue 1.3×10^{-3} m, potassium cyanide 4.7×10^{-3} m; Curve 3, methylene blue 6.3×10^{-3} m, inhibitor 4.75×10^{-4} m; Curve 4, methylene blue 1.3×10^{-3} m, inhibitor 4.75×10^{-4} m; Curve 5, methylene blue 6.3×10^{-3} m, inhibitor 9.50×10^{-4} m. For further details see the text.

The possibility existed that the activation of succinic dehydrogenase by methylene blue was due to a combination of the anionic detergents with cationic methylene blue. To ascertain whether this is actually the case, the effect of two levels of methylene blue was determined (Figs. 3 and 4). If the reactivation of the completely inhibited succinoxidase system by methylene blue were due to a removal of α -TPh or SDS from the enzyme, a 5-fold increase in the methylene blue concentration would be expected to

decrease the degree of inhibition. However, the degree of inhibition of the dehydrogenase by these inhibitors was the same, within experimental error, for both the low and high methylene blue concentrations. The slightly increased oxygen consumption in all the flasks containing the higher level of methylene blue may be ascribed to an increased saturation of the active centers by this hydrogen carrier.

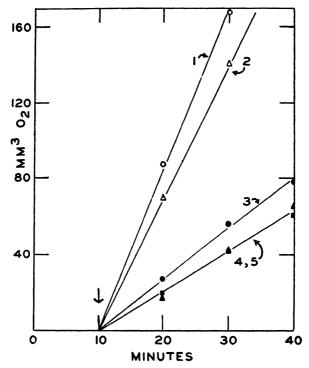


Fig. 4. The effect of methylene blue upon rat heart succinoxidase completely inhibited by SDS. For an explanation of the curves, see Fig. 3.

Interpretation—The partial inhibition of both succinic dehydrogenase and cytochrome oxidase by α -TPh at levels that can completely inactivate the succinoxidase system may be rationalized by the possibility that α -TPh prevents the interaction of the dehydrogenase with cytochrome c. Two mechanisms may be suggested.

 have explained the inhibition of succinoxidase by the antimalarial naphthoquinone SN-5949 and its reactivation by methylene blue to such a mechanism, while Case and Dickens (22) have reported a similar effect produced by 4,4'-dihydroxystilbene.

(2) α -TPh, when absorbed on the dehydrogenase surface, interferes in the association of the dehydrogenase with cytochrome c. It is possible that methylene blue, being a much smaller molecule than cytochrome c, can diffuse past these surface barriers to react with the active centers of the dehydrogenase. Keilin and Hartree (11, 23) and Potter (24) have suggested that the configuration of the dehydrogenase essential for combination with cytochrome c may be altered by methods used in attempts to demonstrate an intermediate.

Indeed, the former authors (11) have recently critically examined several studies in which intermediates linking the dehydrogenase and cytochrome systems were postulated. They presented evidence indicating that in several cases the activity of a partially inactivated succinoxidase system could be increased by addition of denatured proteins or other materials which present a large surface to the reaction medium. The suggestion was made that the colloidal structure of the preparation affects the mutual accessibility of the enzymes in the system.

Interpretations of the effect of inhibitors of the succinoxidase system in terms of biological function should be made with recognition of the lability of this enzyme to a number of different inhibitors. For example, in addition to the inhibition by α -TPh, the succinoxidase system is known to be inhibited *in vitro* by quinones (21, 25), estrogens (26, 22), narcotics and urethanes (23), lacrimators (27), and other —SH reagents (28), fatty acids (29), and by synthetic surface-active compounds (12).

Previous work has demonstrated that the inhibition of the succinoxidase system by α -TPh might be the result of an accumulation of oxalacetate (4), which acts as a potent competitive inhibitor for succinic dehydrogenase (30). Oxalacetate accumulation is not of major importance in the inhibition by α -TPh and SDS reported herein. This is evident from the partial activity of the dehydrogenase component when the succinoxidase system was completely inhibited and also from the fact that heated liver homogenate, which probably contained both DPN and oxalacetate, partially overcame α -TPh and SDS inhibition. In addition, it was found that when α -TPh, in a level just sufficient to cause 100 per cent inhibition, was added after 10 minutes to a normal succinoxidase system, inhibition was complete in another 10 minutes. Since oxygen uptake was normal during the first interval, it does not seem likely that the subsequent inhibition could have resulted from preservation of any remaining DPN.

The similarity and multiplicity of the effects of α -TPh and of other

anions with large non-polar groups do not support the view that the observed effects of α -TPh are a specific function of vitamin E.⁵ It is true, however, that they do not preclude the possibility that tocopherol may function as the phosphate ester or a similar water-soluble derivative.

SUMMARY

Inhibition of the rat heart succinoxidase system by α -tocopheryl phosphate is due principally to the prevention of the interaction of the succinic dehydrogenase with cytochrome c. Lesser inhibitory effects on succinic dehydrogenase and on cytochrome oxidase are also evident.

The effects of α -tocopheryl phosphate on the components of the succinoxidase system are closely paralleled by sodium dodecyl sulfate. Likewise, both substances decrease the characteristic light absorption of reduced cytochrome c.

The results of these studies, together with data already present in the literature, lead to the conclusion that the observed effects of α -tocopheryl phosphate on enzyme systems are due to its properties as an anion with a large non-polar group and are not necessarily related to its action as a vitamin.

BIBLIOGRAPHY

- 1. Houchin, O. B., J. Biol. Chem., 146, 313 (1942).
- Govier, W. M., Yanz, N., and Grelis, M. E., J. Pharmacol. and Exp. Therap., 88, 373 (1946).
- 3. Zierler, K. L., Grob, D., and Lilienthal, J. L., Jr., Am. J. Physiol., 153, 127 (1948).
- 4. Nutr. Rev., 6, 346 (1948).
- 5. Jacobi, H. P., Chappell, J. W., and Morgulis, S., Federation Proc., 6, 136 (1947).
- 6. Spaulding, M. E., and Graham, W. D., J. Biol. Chem., 170, 711 (1947).
- 7. Putnam, F. W., Advances in Protein Chem., 4, 79 (1948).
- 8. Schneider, W. C., and Potter, V. R., J. Biol. Chem., 149, 217 (1943).
- 9. Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 188 (1945).
- 10. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 122, 298 (1937).
- 11. Keilin, D., and Hartree, E. F., Biochem. J., 44, 205 (1949).
- 12. Hockenhull, D., Nature, 162, 850 (1948).
- 13. Boyer, P. D., Ballou, G. A., and Luck, J. M., J. Biol. Chem., 167, 407 (1947).
- Houchin, O. B., in Transactions of 1st Conference on Biological Antioxidants, New York, Josiah Macy, Jr., Foundation, 60 (1946).

⁵ Dr. Stanley Ames, in a private communication, has informed us that he finds that the phosphate esters of γ - and δ -tocopherols have inhibitory effects on the succinoxidase system similar in magnitude to that of α -tocopheryl phosphate, and thus he has concluded that the inhibitory action of α -tocopheryl phosphate is probably not related to the biological function of tocopherol. Dr. Zierler, in a private communication, has also notified us of his conclusion that the *in vitro* action of α -tocopheryl phosphate probably does not reflect the *in vivo* function of tocopherol.

- 15. Keilin, D., and Hartree, E. F., Nature, 145, 934 (1940).
- 16. Sevag, M. G., and Ross, O. A., J. Bact., 48, 677 (1944).
- 17. Hopkins, F. G., Lutwak-Mann, C., and Morgan, E. J., Nature, 143, 556 (1939).
- 18. Stern, K. G., and Melnick, J. L., Nature, 144, 330 (1939).
- 19. Stoppani, A. O. M., Nature, 160, 52 (1947).
- 20. Slater, E. C., Nature, 161, 405 (1948).
- 21. Ball, E. G., Anfinsen, C. B., and Cooper, O., J. Biol. Chem., 168, 257 (1947).
- 22. Case, E. M., and Dickens, F., Biochem. J., 43, 481 (1948).
- 23. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 129, 277 (1940).
- 24. Potter, V. R., J. Biol. Chem., 141, 775 (1941).
- 25. Bartlett, G. R., J. Pharmacol. and Exp. Therap., 93, 329 (1948).
- 26. McShan, W. H., and Meyer, R. K., Arch. Biochem., 9, 165 (1946).
- 27. Mackworth, J. F., Biochem. J., 42, 82 (1948).
- 28. Potter, V. R., and DuBois, K. P., J. Gen. Physiol., 26, 391 (1943).
- 29. Peters, R. A., and Wakelin, R. W., Biochem. J., 32, 2290 (1938).
- 30. Pardee, A. B., and Potter, V. R., J. Biol. Chem., 176, 1085 (1948).

INTRACELLULAR DISTRIBUTION OF ENZYMES

V. FURTHER STUDIES ON THE DISTRIBUTION OF CYTOCHROME c IN RAT LIVER HOMOGENATES

BY WALTER C. SCHNEIDER AND GEORGE H. HOGEBOOM

(From the National Cancer Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, October 12, 1949)

In a previous paper (1) the distribution of cytochrome c in fractions isolated by means of differential centrifugation from suspensions of rat liver in distilled water and in isotonic saline was described. These studies indicated that the cytochrome c of the liver cell was concentrated in the large granule (mitochondria) fractions isolated from these liver suspensions. The results were inconclusive, however, because of the poor yields of mitochondria obtained in these fractionations due mainly to incomplete cell rupture as well as to the agglutination of mitochondria in the saline medium.

The discovery that solutions of sucrose prevented agglutination of mitochondria (2) was coupled with a method that permitted almost quantitative breakage of cells (3) in the development of more efficient methods of liver fractionation (2, 4), and hence a reexamination of the distribution of cytochrome c within the liver cell was indicated. The results of experiments in which the distribution of cytochrome c was determined in fractions isolated from homogenates of rat liver in isotonic and hypertonic sucrose are described in the present report.

EXPERIMENTAL

Materials and Methods

Measurement of Succinoxidase Activity—Succinoxidase activity was determined as described previously (5) by measurement of oxygen uptake in a Warburg apparatus at 38° . Since the rate at which oxygen is absorbed by liver suspensions in the presence of succinate is limited by the amount of cytochrome c present, measurements of succinoxidase activity were made in the absence and in the presence of excess added cytochrome c.\(^1 The results are reported as total and specific succinoxidase activities. The total activities (c.mm. of O_2 absorbed per hour) reported were determined in the presence of excess added cytochrome c. The specific activities

 $^{^1}$ Cytochrome c obtained from the Treemond Company and the Sigma Chemical Company was used.

(c.mm. of O_2 absorbed per hour per mg. of total nitrogen) are reported for measurements made in the presence of added cytochrome c $(Q_{\bullet}^{+}{}^{\circ})$ and in the absence of added cytochrome c $(Q_{\bullet}^{-}{}^{\circ})$.

Determination of Cytochrome c—Cytochrome c was isolated from the liver homogenates and liver fractions and was determined spectrophotometrically as described by Rosenthal and Drabkin (6).

Preparation of Rat Liver Homogenates—Livers were obtained from adult rats (Sprague-Dawley) that had been fasted overnight to remove liver glycogen. Immediately upon removal the livers were chilled over cracked ice and forced through a masher to remove connective tissue. The liver pulp was weighed and homogenized in 9 volumes of ice-cold isotonic (0.25 m) or hypertonic (0.88 m) sucrose in distilled water. The homogenates were forced through a No. 20 gage hypodermic needle and were then fractionated at 0° in the International model PR-1 and in the Spinco model E centrifuges.

Fractionation of Rat Liver Homogenate in 0.88 M Sucrose—20 ml. of homogenate in 0.88 M sucrose were centrifuged 10 minutes at 3000 R.P.M.² (1600 \times g)³ to sediment the nuclei, unbroken liver cells, and red blood cells. The sediment was washed once by resuspending in 8.0 ml. of 0.88 M sucrose and recentrifuging at the same speed. The final sediment was resuspended in 0.88 M sucrose and labeled the nuclear fraction, N_{π} .

The supernatant and washing from the nuclear fraction were centrifuged twice for 10 minutes at 23,000 R.P.M.⁴ (29,000 \times g) to sediment the mitochondria. The sediments were combined and washed twice by resuspension in 8.0 ml. of 0.88 m sucrose and resedimentation for 20 minutes at the same speed. The washed sediment was resuspended in 0.88 m sucrose and labeled the mitochondrial fraction, $M_{\rm w2}$.

The supernatant and washings from the mitochondrial fraction were combined to form S_1 , and 35.0 ml. were centrifuged 60 minutes at 39,460 R.P.M.⁵ (130,000 \times g) to sediment the submicroscopic particles. The submicroscopic particles were washed once by resuspending, diluting to 12.5 ml. with 0.88 m sucrose, and recentrifuging 60 minutes at 50,740 R.P.M.⁶ (148,000 \times g). The washed sediment was resuspended in 0.88 m sucrose and labeled the submicroscopic particle fraction, P_w .

The supernatant and washing from the submicroscopic particles were combined and labeled the supernatant, S_2 .

Fractionation of Rat Liver Homogenate in 0.25 m sucrose—20 ml. of homogenate in 0.25 m sucrose were centrifuged 10 minutes at 2000 R.P.M.² (700 \times g) to sediment the nuclei, unbroken liver cells, and red blood cells. The sediment was washed once by resuspending in 8.0 ml. of 0.25 m su-

² International horizontal rotor No. 269, 16 × 150 mm. test-tubes.

⁸ The g values are given for the center of the tubes used.

crose and recentrifuging at the same speed. The final sediment was resuspended in 0.25 m sucrose and labeled the nuclear fraction, N_w .

The supernatant and washing from the nuclear fraction were centrifuged twice for 10 minutes at 9200 R.P.M.⁴ (5000 \times g) to sediment the mitochondria. The sediments were combined and washed twice by resuspension in 8.0 ml. of 0.25 m sucrose and resedimentation at 20,800 R.P.M.⁴ (24,000 \times g) for 10 minutes. The washed sediment was resuspended in 0.25 m sucrose and labeled the mitochondrial fraction, M_{w2} .

The supernatant and washings from the mitochondrial fraction were combined to form S_1 and 35.0 ml. were centrifuged 60 minutes at 25,980 R.P.M.⁵ (57,000 \times g) to sediment the submicroscopic particles. These particles were washed once by resuspending, diluting to 12.5 ml. with 0.25 m sucrose, and recentrifuging 30 minutes at 50,740 R.P.M.⁶ (148,000 \times g). The washed sediment was resuspended in 0.25 m sucrose and labeled the submicroscopic particle fraction, P_w .

The supernatant and washing from the submicroscopic particles were combined and labeled the supernatant, S_2 .

Results

The results of typical fractionations in 0.88 m and 0.25 m sucrose are given in Tables I and II. The values reported are for 20 ml. of homogenate or an equivalent amount of each fraction.

Cytochrome c—The major portion of the cytochrome c of the liver homogenate was recovered in the mitochondrial fraction, M_{w2} , regardless of whether the fractionation was made in 0.88 m sucrose or in 0.25 m sucrose. Approximately 50 per cent of the cytochrome c of the homogenate was recovered in the mitochondrial fraction in each case. The amounts of cytochrome c associated with the nuclear and submicroscopic particle fractions, N_w and P_w , were very low, and the amounts found in these fractions were, in fact, at the lower limits of the method employed for the determination of cytochrome c. It was not possible to determine cytochrome c in fractions S_1 or S_2 because of the great dilutions of these fractions.

Succinoxidase Activity—The distribution of succinoxidase activity in the rat liver fractions was essentially the same as reported previously (1,2,7,8). The major portion of the succinoxidase activity of the rat liver homogenates was recovered in the mitochondrial fraction regardless of whether the fractionation was made in 0.88 m or in 0.25 m sucrose. It will be noted, however, in contrast to previous reports (1, 2, 7, 8) that only about 80 per cent

 $^{^4}$ International multispeed attachment and rotor No. 295, 19 imes 63 mm. Lusteroid tubes.

⁵ Spinco preparative rotor D.

⁶ Spinco preparative rotor A.

of the succinoxidase activity of the homogenate was recovered in the fractions in both sucrose solutions. The reason for this low recovery is not known but has been obtained repeatedly in this laboratory. Apparently some activator is present in fraction S_1 because (as will be shown in a subsequent publication) when this fraction is added to the mitochondria the succinoxidase activity is augmented, and when all three fractions (N_w ,

Table I

Distribution of Nitrogen, Cytochrome c, and Succinoxidase Activity in Fractions
Isolated From Homogenates of Rat Liver in 0 88 m Sucrose

	Total	Cytoo	chrome c	Succinoxidase activity				
Liver fraction	nitrogen	nitrogen Total F		Total	Q _s ^{+c}	Q_s^-c		
	mg	γ	γ	c mm O2 per hr	c mm O2 per hr per mg N	c mm. O2 per hr. per mg. N		
Liver homogenate	72 0	412	5 7	53,400	740	429		
N _w	9 5	23	2 4	4,500	470	202		
M_{w^2}	16 5	212	12 9	29,780	1810	452		
S_1	48 8			6,340	130			
$\mathbf{P}_{\mathbf{w}}$	14 1	29	2 1					
S_2	32 2				1			

TABLE II

Distribution of Nitrogen, Cytochrome c, and Succinoxidase Activity in Fractions
Isolated from Homogenates of Rat Liver in 0 25 m Sucrose

	Cytochrome c		Succinoxidase activity			
Liver fraction	nitrogen	Total	Per mg N	Total	Q_s^+c	Q_s^-c
	mg	γ	γ	c mm O2 per hr	c mm O2 per hr per mg N	c.mm. O2 per hr. per mg. N
Homogenate	72 9	427	5 9	58,920	820	525
N _w	10 8	43	4 0	7,960	740	356
M_{w2}	16 9	219	13 0	37,080	2200	1010
S_1	47 8			2,420	51	
$P_{\mathbf{w}}$	17 8	8 5	0.5			
S_2	27 5					

 M_{n2} , and S_1) are combined the succinoxidase activity equals that of the homogenate.

The succinoxidase activity in the absence of added cytochrome c (Q_s^{-c}) of the mitochondria isolated from the rat liver homogenate in 0.88 m sucrose was considerably lower than that of the mitochondria isolated from 0.25 m sucrose. Thus the Q_s^{-c} of the mitochondria in 0.88 m sucrose was only 452, whereas in 0.25 m sucrose it was 1010. Since the mitochondria

in both 0.88 m and 0.25 m sucrose contained the same amount of cytochrome c, it is apparent that the cytochrome c associated with the mitochondria in 0.25 m sucrose was much more effective in promoting the oxidation of succinic acid than was the cytochrome c associated with the mitochondria in 0.88 m sucrose.

DISCUSSION

The data presented in Tables I and II clearly show that a major portion of the cytochrome c of liver homogenates is associated with, and concentrated in, the mitochondria isolated from these homogenates. These data provide additional evidence for the hypothesis that the mitochondria are the centers of respiratory activity in the liver cell inasmuch as previous studies have already demonstrated the association of other respiratory enzyme systems with the mitochondria (1, 2, 4, 7-9). The association of cytochrome c with particulate structures such as the mitochondria is of considerable interest because it represents the first instance in which a soluble protein has been demonstrated to be associated with an insoluble portion of the cell. This finding indicates that, although enzymes can be obtained in soluble form from tissues, it does not necessarily follow that the enzymes are present within the cells of that tissue in soluble form.

The cytochrome c not associated with the mitochondria is apparently present in the liver cell in soluble form since only about 10 per cent of the total liver cytochrome c was recovered in the other particulate fractions of the liver homogenate (N_w and P_w). The possibility must, however, be considered that the entire cytochrome c of the intact liver cell is present originally in the mitochondria and partially released during homogenization and fractionation.

SUMMARY

- 1. The cytochrome c content and the succinoxidase activity of fractions isolated by means of differential centrifugation from homogenates of rat liver in 0.88 m sucrose and in 0.25 m sucrose were determined.
- 2. Approximately 50 per cent of the total cytochrome c and 60 per cent of the total succinoxidase activity (determined in the presence of excess added cytochrome c) of the rat liver homogenates were recovered in the mitochondrial fraction isolated in either 0.88 m or 0.25 m sucrose. The biological activity of the cytochrome c present in the mitochondrial fractions was demonstrated by measurement of the succinoxidase activity of the mitochondria in the absence of added cytochrome c.

BIBLIOGRAPHY

- 1. Schneider, W. C., Claude, A., and Hogeboom, G. H., J. Biol. Chem., 172, 451 (1948).
- Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., J. Biol. Chem., 172, 619 (1948).

- 3. Potter, V. R., and Elvehjem, C. A., J. Biol. Chem., 114, 495 (1936).
- 4. Schneider, W. C., J. Biol. Chem., 176, 259 (1948).
- 5. Schneider, W. C., and Potter, V. R., J. Biol. Chem., 149, 217 (1943).
- 6. Rosenthal, O., and Drabkin, D. L., J. Biol. Chem., 149, 437 (1943).
- 7. Schneider, W. C., J. Biol. Chem., 165, 585 (1946).
- 8. Schneider, W. C., Cancer Res., 6, 685 (1946).
- 9. Schneider, W. C., and Potter, V. R., J. Biol. Chem., 177, 893 (1949).

METABOLISM OF INORGANIC NITRITE AND NITRATE ESTERS

II. THE ENZYMATIC REDUCTION OF NITROGLYCERIN AND ERYTHRITOL TETRANITRATE BY GLUTATHIONE

BY LEON A. HEPPEL AND R. J. HILMOF

(From the National Institutes of Health, Bethesda, Maryland)

(Received for publication, October 14, 1949)

The cardiovascular drugs nitroglycerin and erythritol tetranitrate, while stable at 37° in neutral aqueous solution, decompose in the presence of blood and other tissue preparations to yield inorganic nitrite (1-3). The mechanism of this reductive breakdown has remained obscure. Oberst and Snyder (3) found that heating only partially destroyed the capacity of rabbit liver homogenate to form nitrite from nitroglycerin and postulated the existence of both a heat-stable and a heat-labile factor.

An explanation for these observations has been provided by the results of the present investigation. Reduced glutathione (GSH) was found to undergo a spontaneous reaction with nitroglycerin and erythritol tetranitrate to form inorganic nitrite and oxidized glutathione. GSH could be replaced by cysteine or cysteinylglycine, but not by thioglycollic acid. The reduction of the nitrate esters apparently preceded their hydrolysis, because inorganic nitrate did not react with GSH to form nitrite. It appeared likely that nitroglycerin would be reduced to a nitrite ester which would in turn yield inorganic nitrite and glycerol. However, no glycerol could be determined among the reaction products.

The reduction of nitroglycerin and erythritol tetranitrate by GSH was found to be catalyzed by an enzyme occurring in liver and other tissues. It was purified from hog liver acetone powder by means of fractionation with ammonium sulfate and alcohol, and selective elution from calcium phosphate gel. The preparations were specific for GSH and cysteinylglycine; there was no catalysis of the reduction of nitroglycerin by cysteine. For convenience, the term "nitrite-forming enzyme" will be used.

When homogenates of liver were dialyzed, they no longer formed nitrite upon incubation with nitroglycerin, because GSH was removed. Heated homogenates possessed residual activity, even though the enzyme was inactivated. This was due to the fact that the spontaneous reduction of nitroglycerin by GSH took place at a measurable rate in neutral buffered solutions.

Methods

Measurement of Enzyme Activity—An ethanolic solution of nitroglycerin (Lilly) containing 1.6 mg. of the ester was evaporated in 30 ml. test-tubes by means of a stream of air. Then the tubes were packed in ice and the following additions were made: phosphate buffer (0.067 m, pH 7.4) 1.44 ml., GSII (Schwarz, 0.2 m) 0.08 ml., KCN (0.2 m) 0.08 ml., enzyme, and water to make 2 ml. The tubes were placed in a water bath at 37° and samples removed for nitrite analysis at 5 and 10 minutes. Blank tubes were incubated without enzyme and the rate of the spontaneous reaction was subtracted from the total rate. A unit of enzyme activity is defined as that amount catalyzing the formation of 1 μ m of nitrite per hour and specific activity as units per mg. of protein.

Analytical Methods—Inorganic nitrite was determined in HgCl₂ filtrates (4) and inorganic nitrate in phosphotungstic acid filtrates (5). Protein was measured nephelometrically with the Beckman model DU spectrophotometer at 340 m μ (6), by the biuret procedure (7), and with the ultraviolet absorption method of Warburg and Christian (8).

GSH was determined in sulfosalicylic acid filtrates (9). While nitrite interfered with the analysis, this effect could be reduced by adding 1.5 ml. of 0.2 per cent sulfanilamide to 3.0 ml. of incubation mixture just before deproteinization. The recoveries then varied from 67 to 72 per cent, with concentrations covering the range of the data in Table III. Since no way was found to improve this recovery, a correction factor was applied to the analyses.

RESULTS AND DISCUSSION

Non-Enzymatic Reaction between Nitroglycerin and Certain Sulfhydryl Compounds—When nitroglycerin was incubated at 37° in buffered solutions below pH 9, no measurable quantity of nitrite was formed in several hours (Fig. 1, Curve C). However, in the presence of GSH a reaction liberating nitrite took place and its rate increased rapidly as the pH became more alkaline (Fig. 1, Curve A). Almost exactly similar rates were obtained when GSH was replaced by equimolar quantities of cysteine or cysteinylglycine. Thioglycollic acid was inactive. Oxidation of cysteine and GSH during the reaction with nitroglycerin was observed in anaerobic incubations, as indicated by loss of the nitroprusside reaction, decrease in iodine titration, and the appearance of hexagonal crystals of cystine. The oxi-

¹ Prepared from GSH according to Kendall, Mason, and McKenzie (10).

² The crystals had a specific optical rotation $[\alpha]_D^{n}$ of -214° when 26.7 mg. were dissolved in 10 ml. of 1 n HCl. The value for an authentic sample of cystine was $[\alpha]_D^{n} = -206^{\circ}$. Further identification of the crystals was made by application of the Sullivan reaction, by which a cystine content of 95 per cent was indicated.

dized glutathione could be quantitatively reduced with zinc in acid solution.

When inorganic nitrate was incubated with GSH, no measurable amount of nitrite was formed.

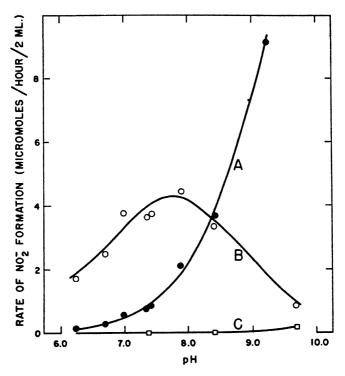


Fig. 1. Formation of nitrite from nitroglycerin and GSH. Curve A, non-enzymatic formation of nitrite from nitroglycerin and GSH. Curve B, rate of the enzyme-catalyzed reaction at different hydrogen ion concentrations. Blank incubation values (without enzyme) were subtracted from the total rate. Curve C, formation of nitrite from nitroglycerin in the absence of both GSH and enzyme. The incubation mixtures contained 78 γ of hog liver enzyme (specific activity 50) and were buffered with 0.05 m phosphate, 0.075 m glycine, or 0.075 m borate.

Enzymatic Reaction between Nitroglycerin and GSH—Nitrite formation from nitroglycerin and glutathione was stimulated when a protein fraction from hog liver was added. Curve B (Fig. 1) shows the rate of the enzymecatalyzed reaction at different pH values. It was obtained by measuring the total rate and subtracting from it the blank rate (without enzyme). The pH optimum is seen to occur between 7 and 8. With cysteinylglycine the rate of the enzyme-stimulated reaction was about one-fourth that ob-

tained with GSH. There was no catalysis of nitrite formation from nitroglycerin and cysteine.

Nitrite-forming activity was found in homogenates of rabbit lung, stomach, uterus, kidney, and liver, and in several organs of the rat which were examined.

Purification of Nitrite-Forming Enzyme from Hog Liver Acetone Powder

Fresh hog liver, kept for several hours on ice, was homogenized in a Waring blendor with 5 volumes of cold (0°) acetone and filtered on a suction funnel. The procedure was repeated on the filtered cake, after which the material was pressed through a wire screen and dried in a current of air. These operations were performed at room temperature. Subsequent

	TABLE I				
Purification of Nitrite-Forming	$Enzyme\ from$	Hog	Liver	Acetone	Powder

Step	Volume	Total units	Over-all yield	Specific activity
	ml.		per cent	unils per mg protein
Crude extract	315	13,010		1.1
1st ammonium sulfate fractionation	133	12,900	99	4.3
2nd " "	85	9,350	72	9.4
Alcohol fractionation	40	4,850	37	37
3rd ammonium sulfate fractionation	35	4,040	31	55
Calcium phosphate, Eluate III	85	1,565	12	80
" " " IV	84	545	4	98

manipulations were at 2°, except as indicated. 56 gm. of acetone powder were suspended in 360 ml. of 0.067 m phosphate buffer, pH 7.4. After a brief interval the mixture was centrifuged for 7 minutes at $13,000 \times g$ (crude extract, Table I).

First Ammonium Sulfate Fractionation—The supernatant was adjusted to pH 5.1 with 1 N acetic acid, an inactive precipitate was removed by centrifuging, and solid ammonium sulfate was added to give 0.5 saturation. This precipitate was rejected, and a second precipitate obtained by increasing the ammonium sulfate concentration to 0.9 saturation was collected, dissolved in 0.067 M phosphate buffer, pH 7.4, and dialyzed 3 hours against running demineralized water.

Second Ammonium Sulfate Fractionation—The pH was again adjusted to 5.1, and the precipitate obtained between 0.6 and 0.8 saturation with ammonium sulfate was collected, dissolved, and dialyzed.

Alcohol Fractionation—The dialyzed ammonium sulfate fraction was diluted with distilled water until the protein concentration was 10 mg.

per ml. Then the pH was adjusted to 5.05 and fractions were collected between the following limits of alcohol concentration: 0 to 0.39, 0.39 to 0.54, 0.54 to 0.65. The solution was kept at -8° as soon as danger of freezing was past. The last precipitate contained the bulk of the enzyme activity, and it was dissolved in 0.067 m phosphate, pH 7.4.

Third Ammonium Sulfate Fractionation—The solution in phosphate buffer was treated directly with solid ammonium sulfate to give 0.6 saturation. The precipitate was rejected and the fraction obtained by increasing the concentration to 0.8 saturation was dissolved in phosphate buffer and dialyzed 4 hours against demineralized water.

Adsorption on Calcium Phosphate Gel—The solution was adjusted to pH 6.3 with dilute acetic acid and diluted to give a protein concentration of 0.5 mg. per ml. To every 10 ml. were added 5 ml. of aged calcium phosphate gel (11) (8.2 mg. dry weight per ml.), after which the gel was eluted successively with 10 ml. portions of M/150, M/90, M/45, and M/15 phosphate buffer, pH 7.4. The last two eluates (Eluates III and IV, Table I) showed the best purification.

All fractions were stable for at least several days in the frozen state. The final preparation was stable for 3 weeks at -15° .

Properties of Nitrite-Forming Enzyme

The rate of nitrite formation was proportional to enzyme concentration. Thus, with an alcohol fraction that had 37 units per mg. the following activities in units per ml. of enzyme solution were obtained with different amounts of material: 0.01 ml., 127; 0.03 ml., 136; 0.04 ml., 135; 0.05 ml., 123; 0.06 ml., 123.

It was felt important to rule out possible catalysis by copper salts. Table II shows that cupric sulfate was actually inhibitory and the enzymecatalyzed reaction was more sensitive than the spontaneous reaction. The enzyme was inactivated by heating to 60°, but was unaffected by 0.1 m sodium fluoride.

The rate of reaction was the same in air and in helium (oxygen-free). A 35 per cent stimulation with 0.008 M cyanide led to its routine use in the enzyme assay. Dialysis for 24 hours against running demineralized water did not alter enzyme activity, nor was the rate of nitrite formation changed by using glycylglycine as a buffer with the omission of phosphate and potassium ions.

Optimal substrate concentrations for the enzyme-catalyzed reaction were 5×10^{-3} m GSH and 3×10^{-3} m nitroglycerin. With excess nitroglycerin the rate was proportional to the concentration of GSH between the limits of 2.5×10^{-4} m and 1.6×10^{-5} m. These observations may provide the basis for a simple and sensitive assay procedure for GSH.

Effect of Cupric Sulfate, Sodium Fluoride, and Incubation at High Temperatures on Reduction of Nitroglycerin by GSH

The incubations in the presence of cupric sulfate were anaerobic, while all other experiments were in an atmosphere of air. Cyanide was omitted from these tests. The incubation mixture contained 78 γ of hog liver enzyme (specific activity 50).

Experimental conditions	Inhibition of enzyme- catalyzed reaction	Inhibition of spontaneous reaction
	per cent	per cent
1 × 10 ⁻³ m cupric sulfate	88	83
1 × 10 ⁻⁴ " "	53	4
1 × 10 ⁻⁵ " " "	40	0
1×10^{-1} " sodium fluoride	0	0
Incubation for 5 mm. at 70°	100	0
" 5 " 60°	50	0

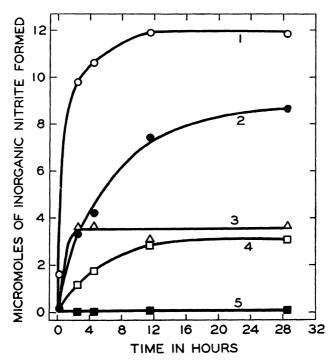


Fig. 2. Formation of inorganic nitrite from nitroglycerin and different amounts of GSH. The incubations were anaerobic, and the usual incubation mixture (see "Methods") was altered by omitting cyanide and increasing the volume to 4 ml. Enzyme addition was 500 γ (specific activity 50). Curve 1, 32 μ M of GSH, with enzyme. Curve 2, 32 μ M of GSH, no enzyme. Curve 3, 8 μ M of GSH, with enzyme. Curve 4, 8 μ M of GSH, no enzyme. Curve 5, no GSH.

The purified fractions were tested in several known systems involving glutathione, but the results were negative. They had no glyoxalase activity, did not catalyze the reduction of cytochrome c by glutathione, and did not stimulate the oxidation of glutathione in air.

Equivalence of Nitrite Formation and GSH Oxidation

Both the rate of nitrite formation from nitroglycerin and the extent of the reaction were limited by the concentration of GSH (Fig. 2). This

TABLE III

Balance between Nitrite Formation and GSH Oxidation upon Incubation of Nitroglycerin and GSH

The incubations were in Warburg vessels, at 37°, in helium. Total fluid volume, 3 ml.; phosphate buffer, pH 7.4, 0.05 m; nitroglycerin, 3.5×10^{-3} m; GSH, 8×10^{-3} m; 430 γ of hog liver fraction (specific activity 50) added as indicated below. GSH tipped in from a side arm at end of gassing period; duplicate flasks incubated, and results averaged.

Experiment No.	Time of incubation	Нq	Enzyme	Glutathione oxidized	Nitrite formed
	hrs.			μМ	μМ
1	11/2	7.0	+	12.4	5.0
	11/2	7.0	_	2.0	0.8
2	11/2	7.4	+	11.3	5.0
3	11	7.4	+	14.1	7.0
4	16	7.4	+	20.2	8.2
	16	7.4	_	12.8	6.3
5	221	7.4	+	16.3	7.6
	221/2	7.4	_	12.0	5.5

suggested that GSH was consumed, which was confirmed by chemical analysis. A balance study was made of GSH disappearance compared with nitrite formation, and the results are shown in Table III. As predicted by theory, 2 μ M of GSH were oxidized for every micromole of nitrite formed.

Dependence of Rate of Nitrite Formation on GSH Content of Tissues

Crude liver preparations formed inorganic nitrite upon incubation with nitroglycerin, but this activity was lost after dialysis. The liver enzyme was not destroyed, because additions of GSH restored the nitrite-forming capacity of dialyzed preparations. The reaction rate varied with the amount of GSH added up to the level of substrate saturation. It then became of interest to compare the chemically determined GSH content of crude liver extract with the amount of GSH required to restore the nitrite-forming capacity completely after dialysis.

The experiment was carried out as follows: Fresh rat liver and hog liver acetone powder were extracted with 0.067 m phosphate buffer, pH 7.4. The extracts were tested for rate of nitrite formation in the presence of nitroglycerin and samples were analyzed for GSH. After being dialyzed for 24 hours against demineralized water, the extracts were nearly completely inactive. Varying amounts of GSH were added, and the amount

TABLE IV

Correlation between Amount of Nitrite Formed by Crude Hog Liver and Rat Liver

Extracts and Their Content of GSH

A 25 per cent homogenate of rat liver and a 7 per cent suspension of hog liver acetone powder, both in 0.067 m phosphate buffer, pH 7.4, were centrifuged. The supernatant was tested for activity in forming nitrite from nitroglycerin. After dialysis for 24 hours against demineralized water, the activity was nearly completely lost, and it was restored by additions of GSH. The amount of GSH required to obtain full initial activity is compared with the GSH content of the undialyzed extracts.

Experiment No.	Extract	GSH content of extract	Additions of GSH	Nitrite- forming activity	Amount of GSH required to restore activity of dialyzed extracts to original level*
		µM per ml.	μΜ	units per ml. extract	µМ рет ті.
1	Hog liver	1.5	1	4.4	1.5
	Dialyzed hog liver	Trace		0.3	
			0.75	3.2	
	" "		1.49	4.1	
	" " "		2.24	5.3	
			2.98	6.1	
2	Rat liver	2.8			3.1
3	Hog "	1.3			0.9
4	Rat "	2.3			2.4

^{*} Obtained by plotting the nitrite-forming activity of the dialyzed extract against the amount of GSH added to it. From this curve one can read the amount of GSH needed to give an activity equal to that of the original extract. The complete data are shown only for Experiment 1.

required to restore the rate of nitrite formation to its original level was determined. This amount agreed fairly well with the GSH content of the extracts before dialysis (Table IV).

Other Reaction Products

The reduction of nitroglycerin by GSH must have preceded hydrolysis, since inorganic nitrate did not undergo conversion to nitrite. The possibility was considered that glyceryl trinitrite was the primary reaction

product. This breaks down spontaneously into inorganic nitrite and glycerol (12). Glycerol and inorganic nitrite were reported formed when nitroglycerin was reduced by aluminum in strong acid (13) and by thiophenol in alcoholic KOH (14).

Samples of the incubation mixture were analyzed for glycerol by means of periodate oxidation (15). None was found. In separate experiments glycerol was added in varying amounts. After an incubation period of 16 hours, samples were analyzed for glycerol, and recovery was complete. Apparently this compound was not formed in the reaction between nitroglycerin and GSH or cysteine. Qualitative tests were made for volatile aldehydes which have been reported as products of nitroglycerin hydrolysis in alcoholic KOH (16). Only traces were found.

Table V
Formation of Nitrite from Erythritol Tetranitrate

tol tetranitrate (Merck) was 0.2×10^{-3} m.

The incubation mixture contained 80 γ of purified hog liver enzyme (specific activity 50). Other additions as described under "Methods," except that, for experiments at pH 9.4, 0.08 m borate buffer was used. The concentration of erythri-

GSH	Enzyme	Hq	Nitrite formed
			μM per kr.
+	+	7.4	3.0
+	_	7.4	0.4
_	_	7.4	0
+	_	9.4	4.6
	_	9.4	0.4

Inorganic nitrate was formed in a concentration one-fifth that of nitrite in the presence of purified enzyme. This resulted from hydrolysis of the ester or secondary oxidation of inorganic nitrite. During the aerobic incubation of liver homogenate with nitroglycerin much of the inorganic nitrite formed would be oxidized by an enzymatic mechanism described previously (17).

Reaction with Erythritol Tetranitrate

Inorganic nitrite was liberated from erythritol tetranitrate in the presence of GSH, and the reaction was catalyzed by the purified hog liver enzyme preparation (Table V). The non-enzymatic reduction of erythritol tetranitrate by glutathione was 10 times as fast at pH 9.4 as at pH 7.4, just as with nitroglycerin. In the absence of glutathione no measurable nitrite was formed when the ester was incubated at pH 7.4 and the rate was very slow at pH 9.4.

SUMMARY

- 1. Nitroglycerin and reduced glutathione (GSH) were found to undergo a reaction forming nitrite and oxidized glutathione. Reduction of nitroglycerin preceded hydrolysis, because inorganic nitrate did not react with GSH. The rate of nitrite formation was appreciable in neutral aqueous solution but increased rapidly as the pH became more alkaline. GSH could be replaced by cysteine or cysteinylglycine.
- 2. An enzyme has been purified from hog liver acetone powder which catalyzes the reaction between nitroglycerin and GSH. It had a pH optimum between 7 and 8 and was sensitive to traces of cupric salts. The enzyme did not stimulate the reaction between cysteine and nitroglycerin.
- 3. When crude tissue extracts were incubated with nitroglycerin, nitrite was formed. The rate and extent of the reaction could be correlated with the GSH content of these extracts.
- 4. Erythritol tetranitrate formed nitrite in the presence of GSH, and this reaction also was catalyzed by the protein fraction purified from hog liver acetone powder.

Miss Virginia T. Porterfield assisted in carrying out some of these experiments.

BIBLIOGRAPHY

- 1. Crandall, L. A., Jr., J. Pharmacol. and Exp. Therap., 48, 127 (1933).
- Yagoda, H., and von Oettingen, W. F., Pub. Health Bull., U. S. P. H. S., No. 282 (1944).
- 3. Oberst, F. W., and Snyder, F. H., J. Pharmacol. and Exp. Therap., 93, 444 (1948).
- 4. Shinn, M. B., Ind. and Eng. Chem., Anal. Ed., 13, 33 (1941).
- 5. Yagoda, II., and Goldman, F. H., J. Ind. Hyg. and Toxicol., 25, 440 (1943).
- 6. Bücher, T., Biochem. et biophys. acta, 1, 292 (1947).
- 7. Weichselbaum, T. E., Am. J. Clin. Path., Tech. Suppl., 10, 40 (1946).
- 8. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941-42).
- 9. Woodward, G. E., and Fry, E. G., J. Biol. Chem., 97, 465 (1932).
- 10. Kendall, E. C., Mason, H. L., and McKenzie, B. F., J. Biol. Chem., 88, 409 (1930).
- 11. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 124, 397 (1938).
- 12. Masson, O., Ber. chem. Ges., 16, 1697 (1883).
- 13. Balatre, P., and Ardaens, A., Ann. pharm. franc., 5, 457 (1947).
- 14. Klason, P., and Carlson, T., Ber. chem. Ges., 39, 2752 (1906).
- 15. Voris, L., Ellis, G., and Maynard, L. A., J. Biol. Chem., 133, 491 (1940).
- 16. Berl, E., and Delpy, M., Ber. chem. Ges., 43, 1421 (1910).
- 17. Heppel, L. A., and Porterfield, V. T., J. Biol. Chem., 178, 549 (1949).

NEW SYNTHESES OF α-AMINO-ε-GUANIDINO-n-CAPROIC ACID (HOMOARGININE) AND ITS POSSIBLE CONVERSION IN VIVO INTO LYSINE*

BY CARL M. STEVENS AND JESS A. BUSH

(From the Fulmer Chemical Laboratory, State College of Washington, Pullman)

(Received for publication, July 14, 1949)

 α -Amino- ϵ -guanidino-n-caproic acid has a certain interest as a homologue of the amino acid arginine. In view of the metabolic rôles already indicated for the homologues of serine (1), cysteine (2), and glutamic acid (3), it is not inconceivable that "homoarginine" may be found to occur naturally. Some interest in the compound derives also from the fact that synthetic homologues of certain biologically active compounds of diverse types have proved to have physiological activities. Since homoarginine is a derivative of lysine, studies of the former might provide some insight into the metabolism of the latter compound.

Homoarginine has been synthesized by Steib (5) and by Greenstein (6) but studies of its metabolism have been confined to *in vitro* tests of the action of arginase. The data obtained are conflicting; Steib (5) and Greenstein (6) observed no detectable cleavage of the compound, while Felix and Schneider (7) reported a 30 per cent yield of urea in prolonged treatment with the enzyme. Since the probable products of the action of arginase on homoarginine are urea and lysine, it occurred to us that cleavage of the compound *in vivo* to a significant extent might be checked by testing its ability to support growth in rats on a lysine-deficient diet.

In the course of this work, more convenient syntheses of pl-homoarginine have been devised, and the synthesis of the l isomer has been accomplished.

EXPERIMENTAL

Synthesis of DL-Homoarginine (Procedure A)—This method is based on the procedure of Greenstein (6), with modifications and involves the fol-

- *Presented in part at the 114th meeting of the American Chemical Society, Portland, Oregon, September, 1948. Supported in part by a grant from the State College of Washington Research Fund.
- ¹ Since this paper was submitted, Dent (4) has suggested that a compound, "fast arginine," detected in certain natural materials by the method of paper chromatography may possess the structure of homoarginine. Our own experiments have indicated that the behavior of homoarginine on paper chromatograms is that of a "fast arginine." It has not yet been possible to make any direct comparison of the materials.

lowing steps: DL-lysine \rightarrow ϵ -carboallyloxy-DL-lysine \rightarrow α -benzoyl- ϵ -carboallyloxy-DL-lysine \rightarrow α -benzoyl-DL-lysine \rightarrow α -benzoyl-DL-homoarginine \rightarrow DL-homoarginine.

 ϵ -Carboallyloxy-dl-lysine—This compound was prepared according to the procedure of Stevens and Watanabe.

α-Benzoyl-ε-carboallyloxy-dl-lysine—By the benzoylation procedure of Carter and Stevens (8), 20 gm. of ε-carboallyloxy-dl-lysine yielded 20 to 21 gm. (69 to 72 per cent) of recrystallized material (from benzene); m.p. 88-91° (uncorrected); N 8.39, 8.27; calculated for $C_{17}H_{22}O_5N_2$, N 8.37 per cent.

α-Benzoyl-DL-lysine—20 gm. of α-benzoyl- ϵ -carboallyloxy-DL-lysine, 200 ml. of glacial acetic acid, 8 gm. of palladium-charcoal catalyst (9), and 4 ml. of concentrated hydrochloric acid were placed in a Parr hydrogenation apparatus and shaken under 20 to 25 pounds of hydrogen for 6 hours. The catalyst was removed, fresh catalyst was added, and the hydrogenation repeated. The final mixture was filtered, the filtrate evaporated to a syrup, the syrup dissolved in water, the solution extracted with ether, and the aqueous phase neutralized with ammonium hydroxide. The yield of product, recrystallized from hot water, was 10 to 10.5 gm. (70 to 73 per cent); m.p. 206° (uncorrected). Greenstein (6) reports the melting point at 211°.

 $\alpha\text{-}Benzoyl\text{-}\text{DL-}homoarginine}\text{--}10~\text{gm.}$ of $\alpha\text{-}\text{benzoyl\text{-}DL-}lysine$ were dissolved in 40 ml. of 2 n sodium hydroxide. S-Methylisothiourea sulfate (5.5 gm.) was dissolved in 55 ml. of warm water. The two solutions were combined (under the hood) and warmed in a water bath at 65–70° for 15 minutes. Crystals frequently separated during this time. The reaction mixture was allowed to cool for a few minutes, a layer of ether was added, and the flask was stoppered and allowed to remain at room temperature overnight.

The aqueous layer was cooled and the precipitate collected and recrystallized from hot water, yielding 5.8 gm. (50 per cent) of pure material melting at 265° (uncorrected); N 18.82, 18.97; calculated for C₁₄H₂₀O₃N₄, N 19.16 per cent. Greenstein (6) reports the melting point at 273°.

DL-Homoarginine Monohydrochloride—10 gm. of α -benzoyl-DL-homoarginine were hydrolyzed under reflux for 3 hours with 100 ml. of 5 n hydrochloric acid. The amino acid was isolated as the monohydrochloride by a procedure similar to that employed for the isolation of arginine from benzylidene arginine (10). The syrupy hydrolysis product dissolved in 40 ml. of 95 per cent ethanol was cooled and treated with 4.5 ml. of freshly distilled aniline. The product which separated on cooling was recrys-

² Stevens, C. M., and Watanabe, R., unpublished.

tallized from a small volume of water by addition of 95 per cent ethanol. The yield was 5.1 gm. (66 per cent) of material melting at 224°.

Analysis—C₇H₁₇O₂N₂Cl. Calculated. C 37.41, H 7.63, N 24.93 Found. " 37.74, " 7.74, " 24.90

Synthesis of DL-Homoarginine (Procedure B)—In this method, the copper complex of lysine is treated directly with O-methylisourea and the homoarginine isolated from the reaction mixture.

5 gm. of DL-lysine monohydrochloride were dissolved in 20 ml. of water and converted to the copper complex by boiling with 2.5 gm. of cupric oxide, and then removing the excess reagent by filtration. The solution was cooled to 25° and added to a solution of 3.3 gm. of O-methylisourea³ in 6 ml. of water. Sodium hydroxide solution (4 N) was added to pH 10.4. The resulting solution was kept at room temperature for 12 hours and then for 24 hours in an ice box. The crystalline precipitate was collected and the filtrate treated with O-methylisourea as before. A much smaller crop of crystals was collected. The combined crops of crystals (4.9 gm.) were dissolved in 50 ml. of warm water and decomposed with hydrogen sulfide. The copper sulfide was removed by filtration with the aid of infusorial earth. The filtrate was acidified to Congo red by the addition of 5 N hydrochloric acid and concentrated to a syrup in vacuo. The homoarginine was recovered as the monohydrochloride and purified as described above. The over-all yield from lysine was 3.0 gm. (48 per cent); m.p. 224° (uncorrected); N 24.72, 24.47 amino N (Van Slyke) 6.6; calculated for C₇H₁₇O₂N₄Cl, N 24.93, amino N 6.23 per cent.

L-Homoarginine Monohydrochloride—This compound was prepared essentially according to Procedure B for the preparation of the DL compound, except that it was found that one treatment with O-methylisourea, with the solution adjusted to pH 10.0 to 10.2, gave a yield of 21 to 24 gm. of homoarginine-copper complex from 20 gm. of L-lysine monohydrochloride. When the syrupy product resulting from decomposition of the copper complex and evaporation of the acidified solution was dissolved in 100 ml. of 95 per cent ethanol and treated with aniline (15 ml.), the crude product separated on prolonged cooling. Addition of an equal volume of ethanol produced a second crop, the total yield being 12 to 16 gm. The material was recrystallized by dissolving it in an equal weight of water and adding 4 volumes of ethanol. An additional crop was obtained by adding more alcohol. The total yield was 10 to 14 gm. (40 to 60 per cent); m.p. 207-

³ Cyanamide was kindly supplied by Dr. J. T. Cassaday, American Cyanamid Company, Stamford, Connecticut. It was recrystallized and then converted to O-methylisourea by the procedure of McKee (11).

209° (uncorrected); $[\alpha]_{D}^{23} = +18^{\circ}$ (1.2 per cent in 1 N hydrochloric acid); N 25.12; calculated for $C_7H_{17}O_2N_4Cl$, N 24.93 per cent.

The preparations of DL-homoarginine used in the feeding experiments were shown to be free of lysine by microbiological assay (12) by using *Streptococcus faecalis* and *Leuconostoc mesenteroides*. Under the conditions of the assay, these organisms were apparently incapable of utilizing homoarginine in a lysine-free medium.

The preparations of L-homoarginine used in the feeding experiments were shown to be free of significant quantities (less than 2 per cent) of lysine by chromatographic examination (13) with paper strips and phenol as the solvent in the presence of ammonia. The L-homoarginine was shown to migrate appreciably faster than L-lysine, the approximate R_F values being 0.87 and 0.74, respectively.

Feeding Experiments—Two series of feeding experiments were conducted. In the first group, the ability of DL-homoarginine to support growth on a lysine-deficient diet was tested. Twenty weanling albino rats (Sprague-Dawley) were maintained in separate cages and fed ad libitum a basal diet consisting of sucrose 72.0, zein⁴ 19.4, finely ground cellulose (Alphacel, Nutritional Biochemicals Corporation) 2.0, salt mixture (14) 4.0, DL-threonine 0.4, DL-tryptophan 0.4, DL-methionine 0.4, L-histidine monohydrochloride 0.4, and DL-valine 0.4 parts respectively. For the first 6 days, all animals were fed the basal diet plus 0.5 per cent of L-lysine monohydrochloride. At the end of this period, they were divided into five groups, each containing two males and two females. The various groups were fed the basal diet, plus the following supplements: 0.5 per cent L-lysine monohydrochloride, 0.25 per cent L-lysine monohydrochloride, basal diet alone, 0.25 per cent L-lysine monohydrochloride plus 1.0 per cent DL-homoarginine monohydrochloride, and 1.0 per cent DL-homoarginine monohydrochloride. Water-soluble vitamins were administered daily as 1 ml. of a solution containing 10 mg. of thiamine, 10 mg. of riboflavin, 10 mg. of niacin, 12 mg. of pyridoxine, 100 mg. of calcium pantothenate, 5 gm. of choline chloride, and 5 gm. of Wilson's (1:20) liver concentrate powder per 500 ml. of solution. 1 drop of halibut liver oil, U. S. P. (Abbott) was given each 4th day. The animals invariably drank the vitamin solutions promptly.

The rats were weighed every 4th day. At that time the food consumed was also measured, and more food was added to the food containers if necessary.

The data obtained in the experiment are recorded in Table I, while the changes in weight of representative animals from each group are shown graphically in Fig. 1. For clarity, and because all groups were remarkably

⁴ Obtained through the courtesy of Dr. A. L. Wilson, Corn Products Refining Company, Argo, Illinois.

uniform, the data for only one animal from each of the groups receiving lysine supplements are plotted. The rats receiving homoarginine did not respond immediately to the compound, but it will be noted that by the end of 12 days they began to show a definite growth effect. At the end of 28

TABLE I
Results of Growth Experiments with DL-Homoarginine on Lysine-Deficient Diet

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		gm.	gm.	per cent
1 ♂	46	1-20	8.5	+2.4	0.5 L-lysine
$2 {\scriptstyle \circlearrowleft}$	43	1-20	8.3	+2.1	0.5
3 ♀	38	1-20	7.5	+2.1	0.5 "
4 ♀	47	1-20	8.8	+2.4	0.5 "
5 ♂	44	1-20	5.3	+0.7	0.25 "
6 ♂	42	1-20	5.0	+0.5	0.25 "
7 ♀	44	1-20	5.8	+0.7	0.25 ''
8 ♀	41	1-20	6.9	+0.9	0.25 "
9 ♂	39	1-28	4.0	-0.2	None
		28-40	3.4	+0.3	1.0 DL-homoarginine
10 ♂	44	1-28	3.5	-0.3	None
		28-40	3.7	+0.1	1.0 pl-homoarginine
11 ♀	50	1-28	4.3	-0.4	None
		28-40	4.2	+0.3	1.0 DL-homoarginine
12 ♀	41	1-28	3.9	-0.2	None
		28-40	3.6	+0.1	1.0 DL-homoarginine
13 ♂	44	1-20	5.1	+0.9	0.25 L-lysine + 1.0 DL-homoarginine
14 ♂	43	1-20	4.8	+0.9	0.25 " $+ 1.0$ "
15 ♀	43	1-20	5.3	+0.9	0.25 " $+1.0$ "
16 ♀	41	1-20	5.3	+0.7	0.25 " $+ 1.0$ "
17 ♂	50	1-28	4.0	+0.1	1.0 DL-homoarginine
		28-40	4.7	0.0	None
18 ♂	41	1-28	4.0	+0.1	1.0 pl-homoarginine
		28-40	5.0	-0.2	None
19 ♀	5 0	1-28	4.2	+0.1	1.0 DL-homoarginine
		28-40	4.5	-0.3	None
20 ♀	42	1-28	4.0	+0.1	1.0 DL-homoarginine
		28-40	4.4	0.0	None

days, this group of rats was placed on the basal diet with no supplement. At the same time, to the diet of the group which had previously been receiving the basal diet without supplement was added 1 per cent of DL-homoarginine. From the chart, it will be noted that the first group promptly ceased growing. The group now receiving homoarginine showed a definite, but delayed, growth response.

In the second feeding experiment an attempt was made to determine whether the growth response to homoarginine was the result of bacterial action in the intestine by a comparison of the response of different groups of animals receiving the compound by mouth and by subcutaneous injection. Also, higher levels of homoarginine were tested to determine whether the

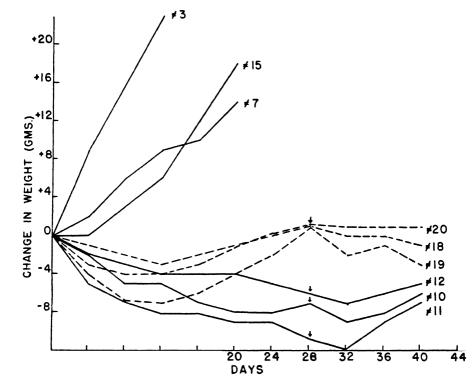


Fig. 1. Changes in weight of rats on a lysine-deficient diet. The small vertical arrows indicate a change of diet. Rats 18, 19, and 20 (broken lines) received 1 per cent DL-homoarginine until the 28th day, when they were placed on the basal diet, while Rats 10, 11, and 12 were placed on the basal diet until the 28th day, when it was supplemented with 1 per cent DL-homoarginine. The remaining rats were fed the following diets throughout: Rat 3, 0.5 per cent L-lysine; Rat 7, 0.25 per cent L-lysine; Rat 15, 0.25 per cent L-lysine plus 1 per cent DL-homoarginine.

growth response could be increased. In these experiments, L-homoarginine was used.

Groups of four male albino rats, of the Sprague-Dawley strain 40 days old, were fed the basal diet used in the previous experiment with the following supplements: 0.75 per cent L-lysine monohydrochloride, 0.75 per cent L-homoarginine monohydrochloride, and 1.5 per cent L-homoarginine mono-

Table II
Comparison of Growth Response from L-Homoarginine When Fed and When Injected

Rat No	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		gm.	gm.	
102	100	1-20	7.6	-0.6	None
118	93	1-20	5.8	-0.7	"
115	87	1-20	6.1	-0.6	"
127	71	1-20	5.6	-0.7	"
109	104	1-16	12.3	+3.7	0.75% L-lysine in diet
112	89	1-16	11.3	+3.6	0.75% " " "
126	90	1-16	11.1	+3.4	0.75% " " "
129	58	1-16	7.5	+2.3	0.75% " " "
110	87	1-20	8.4	+1.2	100 mg. L-lysine per day by subcutaneous
					injection
113	101	1-20	9.8	+1.0	11 11
121	67	1-20	7.5	+1.0	., .,
122	90	1-20	8.5	+1.3	
105	100	1-8	7.2	-0.6	0.75% L-homoarginine in diet
		9-20	9.8	+1.2	0 75% """
111	94	1-8	4.1	-1.5	0.75% " " "
		9-20	6.0	+0.5	0.75% """
120	87	1-8	5.0	-0.9	0.75% " " "
		9-20	6.3	+0.4	0.75% " " "
119	73	1-8	4.0	-1.2	0.75% " " "
		9-20	4.8	+0.7	0.75% " " "
101	73	1-8	4.6	-1.0	100 mg. L-homoarginine per day by injec-
					tion
		9-20	5.3	+0.3	" "
108	96	1-8	6 6	-1.0	" "
		9-20	7.4	+0.2	" "
114	94	1-8	5.8	-1.1	" "
		9-20	6.4	+0.2	" "
123	87	1-8	4.9	-1.1	" "
		9-20	6.3	+0.4	" "
104	96	1-8	5.6	-1.5	1.5% L-homoarginine in diet
		9-20	7.8	+1.1	1.5% " " "
106	86	1-8	5.0	-0.5	1.5% " " "
		9-20	6.3	+0.5	1.5% " " "
107	75	1-8	4.5	-0.9	1.5% " " "
		9-20	5.6	+0.4	1.5% " " "
125	95	1-8	6.5	-1.1	1.5% " " "
4.0-		9-20	7.7	+0.7	1.5% " " "
103	96	1-8	5.5	-1.3	200 mg. L-homoarginine per day by injec-
			1	1	tion

Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
gm		gm.	gm.	
	9-20	7.0	+0.3	200 mg. L-homoarginine per day by injec-
,				tion—Continued
77	1-8	4.0	-1.5	<i>u u</i>
	9-20	6.5	+0.3	11 11
76	1-8	4.1	-0.9	" "
	9-20	4.8	+0.3	" "
95	1-8	5.5	-1.4	" "
	9-20	5.4	+0.9	<i>u</i>
	weight	weight Days	Initial weight Days daily food food intake	Initial weight Days daily food food intake daily weight change

TABLE II-Concluded

hydrochloride. Similar groups of animals were fed the basal diet alone but received by subcutaneous injection twice daily one of the following supplements: 25 mg. of L-lysine monohydrochloride, 25 mg. of L-homoarginine monohydrochloride, and 50 mg. of L-homoarginine monohydrochloride. In all other respects, the experiment was conducted in the same manner as the previous one.

The data obtained in the second experiment are recorded in Table II. Among the animals receiving dietary supplements of L-homoarginine, it will be noted that there was again a striking lack of response during the first 8 days, and then a consistent gain through the last 12 days of the experiment. Doubling the level of L-homoarginine in the diet did not increase the rate of gain significantly. The animals receiving subcutaneous injections of L-lysine showed a reasonable rate of growth. The injection of L-homoarginine at the same level gave no response for the first 8 days and then a consistent though rather small growth response. Again, doubling the amount of L-homoarginine injected caused no increase in the growth response of the animals.

DISCUSSION

The syntheses of DL-homoarginine reported in the present paper appear to present definite improvements over published methods. In Procedure A, the use of the more readily available allyl chloroformate and S-methylisothiourea is advantageous, and, in our hands, the isolation of homoarginine as the monohydrochloride is superior to isolation as the sulfate or nitrate. In convenience and over-all yield, Procedure B is still better. By this procedure, L-homoarginine is also readily prepared.

The response to homoarginine in the growth experiments with rats on lysine-deficient diets is rather unique and puzzling. The growth response

is delayed and quite small, though consistently observed in every one of a total of twenty-four animals. The limited growth is not due to any effect of the D isomer, since L-homoarginine produces a small response also. Apparently it is not the result of inefficient utilization of the material, since higher levels of the compound show practically identical effects. Previous studies of growth response to derivatives of lysine (15) have shown no evidence of such a delayed effect. One reasonable possibility is that an alteration or adaptation in intestinal flora may represent the mechanism of utilization of homoarginine. However, the similar growth response of animals injected subcutaneously with homoarginine renders this explanation quite unlikely.

SUMMARY

Improved syntheses of $DL-\alpha$ -amino- ϵ -guanidino-n-caproic acid ("DL-homoarginine") have been devised. L-Homoarginine has been synthesized for the first time.

Under the condition employed, homoarginine shows a small but definite growth response in rats on a lysine-deficient diet. This is true whether the compound is added to the diet or injected subcutaneously. In both cases, the growth response occurs only after an initial delay of several days.

The authors are indebted to Mr. Gail Stapleton for valuable technical assistance.

BIBLIOGRAPHY

- 1. Teas, H. J., Horowitz, N. H., and Fling, M., J. Biol. Chem., 172, 651 (1948).
- 2. Binkley, F., and du Vigneaud, V., J. Biol. Chem., 144, 507 (1942).
- 3. Mitchell, H. K., and Houlahan, M. B., J. Biol. Chem., 174, 883 (1948).
- 4. Dent, C. E., Biochem. J., 43, 169 (1948).
- 5. Steib, H., Z. physiol. Chem., 155, 292 (1926).
- 6. Greenstein, J. P., J. Org. Chem., 2, 480 (1937).
- 7. Felix, K., and Schneider, H., Z. physiol. Chem., 255, 132 (1938).
- 8. Carter, H. E., and Stevens, C. M., J. Biol. Chem., 138, 627 (1941).
- 9. Org. Syntheses, 26, 77, procedure C (1946).
- 10. Org. Syntheses, coll. 1, 49 (1943).
- 11. McKee, R. H., Am. Chem. J., 26, 209 (1901).
- 12. Henderson, L. M., and Snell, E. E., J. Biol. Chem., 172, 15 (1948).
- 13. Williams, R. J., and Kirby, H., Science, 107, 481 (1948).
- Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., J. Biol. Chem. 138, 459 (1941).
- 15. Neuberger, A., and Sanger, F., Biochem. J., 38, 119, 125 (1944).

THE EFFECT OF BIXIN AND CAROTENE ON THE OXIDATION OF METHYL LINOLEATE

By H. O. KUNKEL AND WALTER L. NELSON

(From the Department of Biochemistry and Nutrition, Cornell University, Ithaca)

(Received for publication, May 21, 1949)

While the coupled oxidation of carotene and oils has been studied in some detail (1-7), the rôle of other carotenoids in oxidizing oils is imperfectly understood. Indications that bixin is oxidatively destroyed in a manner similar to carotene were given in the study of lipoxidase by Sumner and Smith (8). Solutions of bixin were also observed to be vastly more stable than similar solutions of carotene. An alcoholic solution of bixin can be kept on the reagent shelf for months without significant deterioration. It was of interest, therefore, to investigate the destruction of bixin in contact with an autoxidizing or a photooxidixing unsaturated oil such as methyl linoleate and to compare the results with those of similar studies of a system containing the relatively unstable carotene. It was believed that such a study might give added information on the mechanism of similar coupled oxidations.

EXPERIMENTAL

The methyl linoleate used in this study was obtained from the Hormel Foundation. Bixin was prepared by hot acetone extraction of annatto powder (Fisher Scientific Company) according to the method described by Sumner and Smith (8). The carotene (General Biochemicals, Inc.) was a 10 per cent α - and 90 per cent β -carotene mixture from a freshly broken ampul.

All oxidations were allowed to take place in Warburg flasks. In the spectrophotometric studies, the flasks were removed at the appropriate times, the contents were diuted with ethanol, and spectrophotometric readings were taken on the dilluted material in the Beckman spectrophotometer. All spectrophotometric data were calculated on the basis of $E_{\rm lom.}^{1\%}$ of the amount of the carotenoid or the linoleate present in each flask at the beginning of the experiment.

Photooxidation experiments were carried out in a standard Warburg apparatus of the American Instrument Company, modified by G. F. Somers and described by Boyle (9). The water bath was fitted with a plate glass bottom. A series of nine alternating "daylight" and "white" 30 watt fluorescent lamps was mounted approximately 2 inches below the bottom of the water bath. This provided an illumination of about 2000 foot-

candles on the bottom of the manometric vessels. In order to prevent moisture condensation on the bottom of the bath and to dissipate some of the heat given off by the lamps, an electric fan was placed so that the stream of air would blow across the surface of the lamps.

Results

In the first series of experiments, the changes in absorption spectra in the bixin-linoleate system were studied. The destruction of bixin was followed by its main absorption band at $457 \text{ m}\mu$. It was noted that when

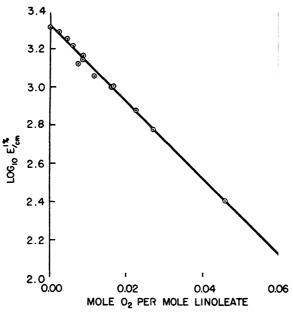


Fig. 1. Logarithm of extinction of bixin (at 457 m μ) compared with oxygen uptake of autoxidizing methyl linoleate. The samples contained 100 mg. of linoleate, 250 γ of bixin, and triacetin to make a total volume of 2 ml. Temperature 25°; gas phase O_2 . Total time for experimental run 72 hours. Extrapolation of the curve to 0.10 mole of O_2 per mole of linoleate gives a value of 1 per cent of the original concentration of the bixin.

10 per cent of the linoleate had been oxidized, only 1 per cent of the original concentration of bixin could be detected. Carotene has been shown by Holman (7) to act in a similar manner. When the logarithm of the bixin concentration is compared with oxygen uptake (Fig. 1), a linear relationship is evident. It appears, therefore, that this coupled oxidation of bixin is a first order reaction with respect to oxygen uptake as compared to a first order reaction with respect to time. These data indicate that the

bixin destruction is a function of oxygen uptake by the linoleate and is probably the result of a reaction with the first oxidation product of the linoleate.

To study the effect of α -tocopherol in the system described above, illumination was used to catalyze the reaction. This allowed a measurable reaction rate without recourse to the addition of catalysts to the flask con-

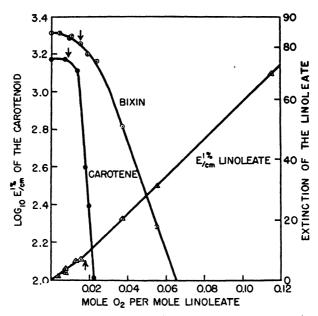


Fig. 2. Logarithm of extinction of bixin (at 457 m μ) and of carotene (at 448 m μ) and absorption of linoleate (at 232.5 m μ) compared with oxygen uptakein photooxidizing tocopherol-methyl linoleate system. The samples contained 100 mg. of linoleate, 250 γ of carotenoid, 125 γ of α -tocopherol, and triacetin to a volume of 2 ml. Temperature 25°; gas phase O_2 ; illumination approximately 2000 foot-candles. The absorption of linoleate was measured in the presence of bixin. Total time for experimental run, 300 hours for the system containing bixin, 100 hours for the carotene system. The arrows indicate approximate points of the termination of the induction period, that is, the beginning of rapid oxygen uptake (see Fig. 4).

tents or to raising the temperature of the reaction. The results of the spectrophotometric studies are shown in Fig. 2. It can be seen that the direct relationship of bixin destruction to oxygen uptake does not hold in the early phases of oxidation. It is when the rate of oxygen uptake abruptly increases (see Fig. 4) that the bixin is destroyed. This destruction is as rapid as in the experiments first described. Carotene measured at 448 m μ exhibits a somewhat similar behavior. While the bixin concentration at the end of the induction period was 90 per cent of the original

concentration, little or no detectable loss in the amount of carotene chromophores was noted. Diene conjugation in the linoleate was measured at 232.5 m μ . It can be seen to occur even during the induction period. Peroxide formation may be assumed to accompany such conjugation of the unsaturated system (10).

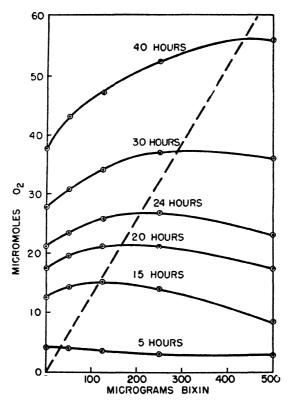


Fig. 3. Oxygen uptake of photooxidizing methyl linoleate in the presence of varying amounts of bixin. The samples contained 100 mg. of linoleate, bixin in the amounts indicated, and triacetin to a total volume of 2 ml. Temperature 25°; gas phase O₂; illumination approximately 2000 foot-candles. Because of the close agreement between duplicates, only average values are plotted. The straight dashed line indicates points of maximal oxygen uptake as the oxidation proceeds.

Preliminary experiments with photooxidation of bixin in dilute methyl linoleate solutions indicated that coupled oxidation of bixin yields a final product which has an absorption maximum at 267 m μ . Attempts to detect intermediates spectrophotometrically during the early phase of oxidation did not give any positive results other than indication of an indefinite transitory increase in absorption between 300 and 315 m μ .

Heftman (1) and Monaghan and Schmitt (5) have reported that small amounts of carotene in an oxidizing system appear to inhibit the oxidation of an unsaturated oil. Increased amounts caused increased oxidation. Ramaswamy and Banerjee (11) have shown that large amounts of annatto powder, which contains bixin in high concentration, exhibit prooxidant activity. In view of this, the effect of varying concentrations of bixin, without tocopherol, on the rate of oxidation of linoleate was studied. In experiments in the dark or dim light, small amounts, 250γ or less of bixin,

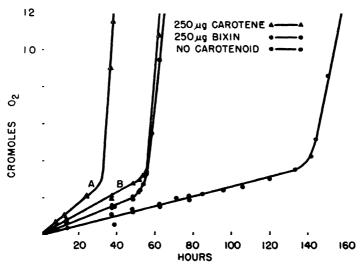


Fig. 4. The effect of carotene and bixin on the rate of photooxidation of methyl linoleate in the presence of α -tocopherol. The samples contained 100 mg. of methyl linoleate, 250γ of carotenoid as indicated, 100γ of α -tocopherol, and triacetin to a volume of 2 ml. Temperature 25°; gas phase air; illumination approximately 2000 foot-candles. Curve A for carotene represents the usual oxygen uptake; Curve B represents an example of a slower rate of oxidation during the induction period.

added to 100 mg. of linoleate, at times exhibited an antioxidant effect in that the rate of oxidation was decreased. Upon repeated experiments, a great variation in results was obtained and these data do not allow any definite conclusions. Above the 250 γ level, however, there seems to be a definite inclination of increased amounts of bixin to cause greater oxygen uptake by the system. At 48 hours, the oxygen uptakes of 100 mg. samples of methyl linoleate with 0, 500, 750, and 1000 γ of added bixin were 0.9, 3.6, 4.0, and 7.0 μ m of oxygen respectively. Here the relative rates of oxidation were established in the early phases and did not later change.

Experiments on the effect of varying amounts of bixin were repeated in the illuminated Warburg apparatus in which a more rapid oxidation was initiated. In the early phases of oxidation under the influence of light, bixin showed a somewhat different action. The larger amounts of bixin actually caused a decrease in the rate of oxidation in the early stages of the reaction. As the reaction proceeded, the increased amounts of bixin then produced a latent acceleration in the rate of oxygen uptake. Representative results are shown graphically in Fig. 3. These effects are highly significant statistically ($P = \langle 0.01 \rangle$) when 500 γ or more of bixin are present in the system. In the oxidation without lights, the larger amounts of bixin established a prooxygenic activity in the very early phases and this pattern showed no later change.

The rate of oxidation of linoleate with α -tocopherol in the system is also affected by the presence of a carotenoid. Fig. 4 represents an experiment showing the effect of carotene and bixin at the 250 γ level. In all cases, carotene caused a significant increase in the oxidation during the induction period. The length of the induction period was usually shorter than that of the system containing no carotenoid, but in a few cases the times were about the same. Bixin usually produced a slower rate of oxidation but always a greatly extended induction period.

DISCUSSION

The antioxygenic effect of bixin in a photooxidizing system indicates that it may stop the reaction chain as proposed for autoxidizing linoleate (12, 13) by supplying a hydrogen atom without accepting one from linoleate or by an addition of the free radical peroxide to a double bond in the carotenoid. An addition reaction is quite possible, for the well known polymerization tendency in autoxidation of olefinic compounds has been explained by such a mechanism (14).

If the donation of a hydrogen atom occurs, it does not necessarily infer the ensuing destruction of the chromophores. The destruction of both carotene and bixin is delayed in the presence of tocopherol. If addition at the double bond occurs, it would disrupt the conjugated double bond system of the carotenoid. This reaction, therefore, would appear to be inhibited in the presence of tocopherol.

The coupled oxidations of bixin and of carotene in oxidizing oils present certain similarities. Both are largely destroyed in the very early phases of the oxidation. Both carotenoids are protected to some extent by the presence of tocopherol. It is interesting to note the different effects of the relatively labile carotene and the more stable bixin in photooxidation of the tocopherol-linoleate system (Fig. 4). Bixin acted synergistically with the tocopherol to enhance its antioxidant activity. The action of carotene is more difficult to interpret. The effect on the rate of oxidation was a stimulatory one, but the length of the induction period was not consistently shorter than in the solution containing no carotenoid.

Monaghan and Schmitt (5) have obtained evidence to suggest that the prooxygenic activity of carotene is the result of products of decomposition. In the experiments reported here, it may be that some degradation occurred in making up the carotene solution and hence would account for the prooxygenic effect. It is also probable that the carotene was activated by absorbing light energy and that the activated molecule in some manner accelerated the reaction.

The experiments with bixin also indicate that the acceleration in the rate of oxidation is caused by the products of degradation. The relative anti-oxygenic activity of bixin and carotene appears to depend on the initial rate of oxidation as well as the presence of tocopherol and the concentration of the carotenoid. In the experiments without light, decomposition products appear to exert their influence to a greater degree and the stopping of the chain oxidation by the unaltered carotenoid does not appreciably affect the rate. If the coupled oxidation is catalyzed by light or by lipoxidase (12), the reactions with the unaltered bixin or carotene slow down the oxidation and the effects of the reaction products are delayed.

It appears that, in coupled oxidation, the carotenoid first reacts either by supplying a hydrogen to a free radical peroxide or by adding to the free radical. The secondary reactions appear to produce highly unstable intermediates which may serve in the perpetuation or initiation of chain reactions. The accumulation of such intermediates would therefore not be expected. This is in agreement with the observations in this study and those of Holman (7) in that no such intermediates could positively be detected spectrophotometrically.

The authors' thanks are due to Dr. G. F. Somers of the United States Nutrition Laboratory at Cornell University for the use of the Warburg apparatus modified by him, and to Dr. R. T. Holman of the Agricultural and Mechanical College of Texas for his helpful suggestions in the early phases of this study.

SUMMARY

The relation of spectral changes to oxygen uptake in the coupled oxidation of bixin and methyl linoleate dissolved in triacetin was studied. Bixin is nearly completely decolorized before 10 per cent of the ester is oxidized. The destruction of bixin proceeds as an apparent first order reaction with respect to oxygen uptake as compared to a first order reaction with respect to time. No definite intermediate decomposition products could be detected.

The presence of α -tocopherol inhibits decolorization of both bixin and carotene until the end of the induction period, after which destruction of the carotenoid rapidly takes place.

An antioxygenic effect is exhibited by bixin in the early phases of the light-catalyzed oxidation of linoleate. Also, bixin acts synergistically with α -tocopherol to enhance its antioxidant activity and produces a markedly extended induction period. This activity appears to be caused by the unaltered carotenoid. A latent acceleration of the reaction without tocopherol appears to be the result of the action of decomposition products of bixin.

In photooxidation of the tocopherol-linoleate system, carotene produces an increased rate of oxygen uptake during the induction period.

BIBLIOGRAPHY

- 1. Heftman, E., J. Am. Oil Chem. Soc., 24, 404 (1947).
- 2. Bradway, E. M., and Mattill, H. A., J. Am. Chem. Soc., 56, 2405 (1934).
- 3. Olcovich, H. S., and Mattill, H. A., J. Biol. Chem., 91, 105 (1931).
- 4. Thompson, C. R., and Steenbock, H., Arch. Biochem., 4, 15 (1944).
- 5. Monaghan, B. R., and Schmitt, F. O., J. Biol. Chem., 96, 387 (1932).
- Holman, R. T., Transactions of the First Conference on Biological Antioxidants, Josiah Macy, Jr., Foundation New York, 37, Oct. (1946).
- 7. Holman, R. T., Arch. Biochem., 21, 51 (1949).
- 8. Sumner, J. B., and Smith, G. N., Arch. Biochem., 14, 87 (1947).
- 9. Boyle, F. P., Thesis, Cornell University (1947).
- 10. Holman, R. T., and Burr, G. O., J. Am. Chem. Soc., 68, 562 (1946).
- Ramaswamy, T. S., and Banerjee, B. N., Ann. Biochem. and Exp. Med., India, 8, 55 (1948).
- 12. Bergstrom, S., and Holman, R. T., Advances in Enzymol., 8, 425 (1948).
- 13. Bolland, J. L., Proc. Roy. Soc. London, Series A, 186, 218 (1946).
- Price, C. C., Mechanisms of reactions at carbon-carbon double bonds, New York (1946).

THE EFFECT OF ZIRCONIUM AND SODIUM CITRATE ON THE DISTRIBUTION AND EXCRETION OF SIMULTANEOUSLY INJECTED THORIUM AND RADIOSTRONTIUM

By JACK SCHUBERT AND HERSCHEL WALLACE, JR.

(From the Medical Division, Argonne National Laboratory, Chicago)

(Received for publication, June 29, 1949)

The potential health hazards of radioelements taken into the body are well known (1, 2). The present study is part of a program designed to test the effect of zirconium and other salts on the metabolism of long lived radioelements. It has been shown that the early administration of zirconium salts to experimental animals previously injected with plutonium (Pu^{239}) and radioyttrium (Y^{91}) resulted in a marked increase in the urinary excretion of these radioelements and a considerable reduction of their concentration in bone (3, 4).

Thorium (Th²³⁰) and radiostrontium (Sr^{89, 90}) are of particular interest because of the marked differences in the nature of their deposition in bone (2). Strontium is closely related metabolically to calcium and the other alkaline earths (2) and is deposited throughout the mineral structure of bone, while many elements, including thorium, plutonium, zirconium, and the rare earths, are deposited primarily in the non-mineralized areas and possibly on the superficial surfaces of the mineralized structure (1, 2). Conditions that affect the metabolism of Ca and Sr do not appear to disturb that of Pu or Y (1, 2). It appeared unlikely, therefore, that zirconium administration would affect the metabolism of Sr.

Until recently there has been little information available on the quantitative distribution and excretion of soluble thorium salts following parenteral administration (2, 6). It has been stated (2) that tracer studies and bone radioautographs showed "no significant differences between the metabolic characteristics of thorium and plutonium." Therefore, it seemed reasonable to expect that the metabolism of injected thorium followed by zirconium administration should be markedly altered, as is the case with plutonium. It is of fundamental interest, therefore, to learn that zirconium has no significant influence on Th metabolism.

Inasmuch as the citrate salt of zirconium was used in our experiments, it was necessary to study the effects of sodium citrate as a control for the action of the citrate ion. Sodium citrate administration is of value in the treatment of experimental uranium poisoning (5) and also of lead poisoning (7), but it exerts little influence on the metabolism of plutonium and yttrium (4).

EXPERIMENTAL

General Procedures-All injections were made by the intraperitoneal route. Twenty female Sprague-Dawley rats, having weights which fell between 197 and 215 gm., were injected with 1.4 ml. of a solution containing the radioisotopes of Th²³⁰ and Sr^{89, 90}. The twenty rats were divided into five groups. To the first group of four rats were administered 4.0 ml. of a zirconium citrate solution containing 12.5 mg. of Zr per ml. ½ hour after injection of the radioelements, while another group of four rats received the same amount of zirconium citrate 3 days later. These groups are referred to as "early zirconium-treated" and "late zirconium-treated," respectively. In similar fashion a third group of four rats received 4.0 ml. of a 5 per cent solution of sodium citrate \frac{1}{2} hour after the injection of the radioelements, and another group of two rats received the same amount of sodium citrate 3 days later. These groups are referred to as "early citrate-treated" and "late citrate-treated," respectively. The last group of six rats, the "untreated controls," received only the radioelement solution. The rats were kept two to a cage. The pooled urine and feces from each cage were collected separately, usually at daily intervals. 8 days after the injection of Th²³⁰ + Sr^{89, 90} the animals were sacrificed with nembutal, and the liver, pancreas, femur, kidneys, spleen, mesenteric lymph node, and the lungs were removed. The rest of the animal constituted the "carcass" portion.

Radioelement Solution—A small volume of a hydrochloric acid solution of the radioelements was added to a solution of citric acid, the pH was adjusted with NaOH, and the solution diluted with distilled water. After dilution the solution used for injection had the following composition: pH 4, citric acid 1 per cent, Na⁺ 0.15 m, Sr^{89, 90} ~15 μ c. per ml., and Th²³⁰ ~0.64 μ c. per ml. Each animal received 1.4 ml. of this solution or a total of 1.4 × 10⁶ counts per minute of Sr^{89, 90} and 0.9 × 10⁶ counts per minute of Th²³⁰. Inasmuch as the specific activity of Th²³⁰ is about 43,000 disintegrations per minute per microgram or 21,500 counts per minute per microgram at 50 per cent geometry, each rat received a minimum of 0.9 × $10^6/21.5 \times 10^3 = 42 \gamma$ of thorium. Chemical analysis revealed the presence of about 70 γ of Th. This concentration is far below the LD₅₀ value (8). A spectrographic analysis of the injection solution showed that, per ml., it contained less than 2 γ of chemical strontium or of cations other than sodium and thorium.

Zirconium and Sodium Citrate—The solution of zirconium citrate, $Na^+[ZrO(C_6H_5O_7)]^-$, was prepared as described previously (4). Each animal receiving zirconium was given 4.0 ml. of a 12.5 mg. per ml. solution or a total of 50 mg. as Zr. The sodium citrate-treated rats each received

4.0 ml. of a 5 per cent solution of sodium citrate, Na₂C₆H₅O₇·2H₂O. The amounts of Zr or sodium citrate administered were well below the toxic limits (8, 9).

Methods of Analysis—The excreta and tissues were wet ashed by the use of concentrated HNO₃ only. This was accomplished by covering the sample with HNO₃ and evaporating to near dryness on a hot-plate. Fresh acid was then added and evaporated as before. This was repeated from three to eight times, depending on the size of the sample, until no organic matter remained, as evidenced by the light yellow to white color of the ash. Finally, the residue was transferred with HNO₃ to a volumetric flask and diluted to volume (usually to 100 ml. for tissues and to 2000 ml. for carcasses). The carcasses were dissolved by first being covered with concentrated HNO₃, allowed to stand for at least 12 hours, and then treated as described above.

The amount of each radioelement injected into each rat was sufficiently high so that a quantitative measure of its concentration in a sample could be made by direct deposition and subsequent counting of the radiations. Inasmuch as Th²³⁰ is primarily an α emitter (10) and Sr^{89, 90} a β emitter (10), it was not necessary to effect any separation. For counting Th²³⁰ an aliquot (usually 0.25 ml.) of an ashed solution was deposited on a platinum disk, slowly evaporated to dryness by means of an infra-red lamp, and flamed. The activity was determined by counting at about 50 per cent geometry in a proportional α -counter unresponsive to β -particles. Samples (usually 5 ml.) for Sr^{89, 90} assay were deposited in a 10 ml. porcelain capsule. evaporated to dryness with an infra-red lamp, and counted by means of a mica window Geiger tube through a 110 mg. per sq. cm. aluminum absorber. The absorber was used to eliminate activity contributed by the accompanying Th²³⁰ which would otherwise be recorded. The samples were not assayed for Sr^{89, 90} until the Sr⁹⁰ isotope had regained equilibrium with its 65 hour Y90 daughter.

The activity in a given sample was always referred to that of samples from the original injection solutions obtained from "dummy" injections into a volumetric flask, which were subsequently mounted and counted under identical conditions. In order to cancel effects due to absorption and other factors, a separate standard for each tissue was prepared by addition of an equivalent amount of an ashed solution of the same tissue from a non-radioactive animal to a sample from the "dummy" injection.

Results

Comparison of Distribution and Excretion of Th²⁰⁰ and Sr^{89, 90} in Untreated Rats—The simultaneous administration of the two radioelements permits a direct comparison of their behavior in the same animal. The

data tabulated in Table I illustrate the striking differences in the excretion and distribution of Th and Sr after 8 days. Sufficient time having elapsed for the uptake in bone to have reached a maximum (2, 11), it was found that the concentration of injected Th was considerable in all the soft tissues, reaching 30 per cent of the injected dose in liver alone, while that of Sr was negligible, *i.e.* less than 0.02 per cent. On the other hand, the concentration of Sr in the bone was 3 times higher than that of Th.

The total urinary and fecal excretion of Sr was about twice that of Th. Urinary excretion of both radioelements was highest during the first 24 hours after injection. It then dropped sharply during the subsequent days, especially in the case of thorium. By the 8th day the urinary excre-

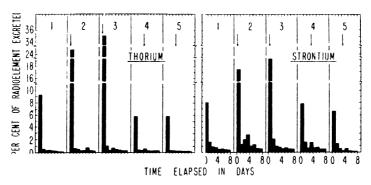


Fig. 1. The effect of zirconium and sodium citrate administration to rats on the urinary excretion of injected Th²³⁰ and Sr^{89,90}, respectively. Each arrow represents a single intraperitoneal injection of zirconium or sodium citrate. The numeral within each panel represents the following treatments: Panel 1 controls, Panel 2 early zirconium citrate, Panel 3 early sodium citrate, Panel 4 late zirconium citrate, Panel 5 late sodium citrate.

tion of strontium had reached about 0.4 to 0.5 per cent of the injected dose per day, while that of thorium had diminished to about 0.05 per cent. The day by day urinary excretion of these elements is shown in Fig. 1. The daily fecal excretion of Sr was quite erratic, while that of Th proceeded at a fairly constant rate of about 0.5 to 1.5 per cent a day.

Effect of Treatment—The early administration of either zirconium or sodium citrate caused the urinary excretion of Th to increase from about 9 to about 30 per cent of the injected dose (Fig. 1) and that of Sr from 8 to about 18 per cent during the subsequent 24 hour period (Fig. 1). Some sustained increase in Sr excretion following early Zr administration, however, seems to have occurred. Injections of sodium citrate 72 hours after exposure to Th and Sr had no effect on their urinary output, while zirconium citrate appeared to have a slight effect on Sr excretion and none on Th (Fig. 1). It must be kept in mind, however, that a 3-fold increase, for

required to bring them into uniform suspension. In the cultures in flasks, no trouble was experienced from pellicle formation. Data to illustrate the stimulation of growth by pimelic acid and by biotin are shown in Table I. These readings were taken after 2 days of incubation. In order to eliminate variations in the results which arose from failure to obtain uniform suspensions of the organism for inoculation, the inoculum was prepared as follows. The cells from 1 cc. of a 1 day-old culture in a 1 per cent tryptose-glucose medium were collected and washed twice. They were then resuspended uniformly by mixing with 1 cc. of sterile saline and grinding briefly in a TenBroeck glass grinder. The suspension so obtained was free of clumps and was diluted 1:100 and 1 drop of this dilution was used per flask or per tube in the tests.

Table I

Growth Response to Brotin and to Pimelic Acid of B. tenuis Cultured in Test-Tubes
and in Flasks

Addition to basal medium	Turk	Turbidity*			
Addition to base medium	In tubes	In flasks			
None	84	65			
Biotin, 0 001 γ per cc	63	61			
" 0.0003 " " "	64	63			
" 0.0001 " " "	88	67			
Pimelic acid, 10 y per cc	75	į			
	71	64			
" " 1 " " "	81	62			

^{*} Expressed as per cent of incident light transmitted by the culture when compared to uninoculated medium.

From the data in Table I it is clear that pimelic acid can replace biotin, but that much more of the former than of the latter was required. The same situation has been shown with the diphtheria bacillus (4).

Because of the more rapid growth obtainable in flasks, all subsequent trials with *B. tenuis* were conducted in flasks, even though the stimulation by biotin and pimelic acid was not then demonstrable.

Inhibition of Growth of B. tenuis by ϵ -(2,4-Dichlorosulfanilido)-caproic Acid—The data in Table II will show that small amounts of this sulfanilide inhibited the growth of B. tenuis under the conditions just described.

Antagonism between ϵ -(2,4-Dichlorosulfanilido)-caproic Acid and Pimelic Acid—The amounts of the analogue needed to cause half maximal inhibition of the growth of B. tenuis were determined in the presence of no added pimelic acid, and with 1 γ of it per cc. and with 3 times this quantity. The results are shown in Table III where it can be seen that pimelic acid

INSTITUTE LIBRARY

Date of leave,	Date of issue	Date of issue,
APP 1954		1
27.6-56	-	
4.7.63,		·
# 5 FEB 19	72	
,		
		4
ļ.		79 .
	<u>.</u>	-
		_

P. P. Works 1,12/48 PJ (ACP) F. 17/595 19/1/48—1002,

example, in the urinary excretion of a radioelement shortly after exposure usually constitutes an appreciable fraction of the body content but is relatively unimportant when the normal excretion rate has dropped to very low levels.

The excretion of Sr or Th in the feces was unaffected at any time by the administration of zirconium or sodium citrate.

Since the major part of the increased excretion of the thorium which followed either early sodium or zirconium citrate administration came from the liver, it must be concluded that the citrate ion is responsible for the observed effects (Fig. 2 and Table I). Thorium excretion was also accomplished at the expense of the other soft tissues, namely the kidneys, spleen,

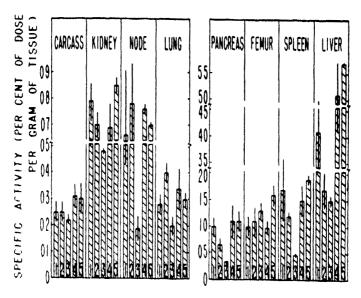


Fig. 2. The effect of zirconium and sodium citrate administration on the concen-

jection of Pu raised the urinary excretion level from 0.1 to 5 per cent of the injected dose.

Table I

Effect of Zirconium and Sodium on Distribution and Excretion of Simultaneously

Injected Thorium and Radiostrontium in Rats*

			Pe	r cent inje	cted dose	e per organ				
Tissues		reated atrols		irconium, -treated		sodium, e-treated		rconium, -treated		sodium, e-treated
	Th	Sr	Th	Sr	Th	Sr	Th	Sr	Th	Sr
Carcass	47.4	67.0	48.2	53.0	41.0	56.9	51.1	66.0	49	70.5
	± 6.9	± 5.4	± 6.2	±4.4	± 2.8	± 9.9	± 9.2	±4	±6	± 3.3
Skeleton†	17.8	52.8	20	40.8	22	44.6	17.2	46.6	28	50
	± 3.6	±6	± 2	± 4.1	±4	± 10	±1.8	± 2	±1	± 0.2
Liver	29.6	0.015	14.4	0.020	9.8	0.010	31.8	0.018	33.8	0.020
	± 6.8	± 0.002	± 2.3	± 0.006	± 2.0	± 0.001	± 4.5	± 0.002	± 1.8	± 0.002
Kidneys	1.2	< 0.02	1.1	< 0.02	0.74	< 0.02	1.0	< 0.02	1.2	< 0.02
	± 0.08	l	± 0.12	1	± 0.12		± 0.17		± 0.1	
Spleen	1.0	< 0.01	0.66	< 0.02	0.27	< 0.01	0.85	< 0.02	0.82	< 0.02
-	± 0.2		± 0.02	1	±0.01		± 0.12		± 0.06	
Mesen-	0.30	< 0.01	0.41	< 0.01	0.12	< 0.01	0.29	< 0.01	0.26	< 0.01
teric	± 0.06		±0.10		± 0.08		± 0.02		± 0.20	
lymph node				1	· !					
	1.0	< 0.01	0.61	<0.01	0.33	< 0.01	1.1	< 0.01	0.93	< 0.01
1 ancicas	±0.2		± 0.10		± 0.08	10.02	± 0.3	10.02	±0.20	
Lung		< 0.02		< 0.02	0.29	< 0.02		< 0.02		< 0.02
246	± 0.04		± 0.04		±0.04		± 0.10		± 0.09	
Urine	10.6	13.5	27.4	27.1	36.8	25.1	6.3	14.2	6	9.70
011110	±4	+4	±6	±8	±3	±5		±0.5	±3	±4
Feces	7.7	16.7	5.5	19.0	9.6	16.2	6.2	17.5	6	17.4
1 0005	±2	± 1.5	± 2	± 2.1	±3	± 2.2	±2	±0.6	±3	±3

^{*} Values equated to 100 per cent (actual recoveries for Th = 94.5 ± 8.2 per cent, and for Sr = 90 ± 4 per cent). The rats were sacrificed 8 days after the intraperitoneal administration of Io^{250} (Th) and Sr^{89} . So. Each value is an average of results obtained from six rats for the untreated controls, four rats each for the early sodium citrate, early zirconium citrate, and late zirconium citrate-treated animals, and two rats for the late sodium citrate group. Further details are given in the experimental sections of the text.

† The concentrations of radioelement in the skeleton were calculated by multiplying the femur content by the somewhat arbitrary factor of 20.

DISCUSSION

Action of Sodium Citrate—The action of early sodium citrate administration in minimizing the deposition of thorium in the soft tissues with a resulting increase in the urinary excretion is related to the complexing action of the citrate ion accompanied by a concomitant increase in the

diffusibility of the Th. Most cations in the body exist as a mixture of diffusible and non-diffusible forms. The non-diffusible fractions result from a combination of the cation with proteins, or because of the formation of particles with colloidal properties as a consequence of hydrolysis or reaction with anions to form salts of low solubility. An increase in the diffusible fraction at the expense of the non-diffusible parts is reflected in a lower deposition in the soft tissues comprising the reticuloendothelial system and an increase in the urinary excretion because the rate of filtration through the glomerulus of the kidney is enhanced. These considerations are supported by the fact that citrates form soluble complex ions with thorium (13) and by ultrafiltration studies in which the passage of Th from blood serum through Visking membranes was markedly increased following the addition of sodium citrate. Similar concepts have been proposed to explain the action of sodium citrate and other complexing axions in the metabolism of uranium (14, 15) and lead (16).

Once an element such as thorium, which precipitates from simple inorganic solution above a pH of 3.5, has been deposited in the body, it is very difficult to increase its subsequent rate of elimination. This is probably related to the well known chemical fact that very large (unphysiological) amounts of the citrate anion are needed to dissolve the hydroxide of an element such as Th once it has formed. This phenomenon explains the relative ineffectiveness of sodium citrate in promoting the excretion of Th when administered several days later.

Of further interest is the fact that the concentration of injected Th in the spleen, mesenteric lymph node, and pancreas was clearly unaffected by early zirconium citrate administration; yet early sodium citrate administration caused a marked reduction. Such a result is probably related to the fact that in zirconium citrate the citrate part of the molecule is not immediately available because it is bound to zirconium. Thus in the time necessary for the zirconium citrate to be absorbed following intraperitoneal injection, the mesenteric lymph node, spleen, and pancreas are not in contact with free citrate. Subsequent metabolic processes free the bound citrate, whereupon it is available to exert a solubilizing effect through complexing action on the Th. Free citrate, however, is lost in the liver partially by conversion to glycogen (17).

The uptake of strontium and the other alkaline earths as well as the phosphate ion is most easily interpreted as being an ion exchange reaction (18–20) with the bone acting as either an anion or cation exchanger containing a variety of functional groups. We can represent the cation exchange with strontium by the relation

$$CaA + Sr^{++} \rightleftharpoons SrA + Ca^{++} \tag{1}$$

¹ Unpublished experiments.

in which, for simplification, we assume that A is a composite anion of all the functional groups in bone with a valence of -2. The capacity of bone for cation exchange can be expressed in terms of the total milliequivalents of replaceable calcium. The distribution coefficient, K_d , for a given cation adsorbed by bone is given by the expression (21)

$$K_d = \frac{M_*}{M_1} \times \frac{v}{m} \tag{2}$$

in which M_s and M_1 are the fractions of the adsorbed cation in the bone and liquid phases respectively, and v is the volume of solution in contact with m mg. of bone. The ratio $M_s:M_1$ is expressed conveniently as (per cent adsorbed)/(100 per cent minus per cent adsorbed). By employing the same reasoning used with resinous cation exchangers (21), it can be shown that, to a first approximation, K_d is directly proportional to the mole fraction of exchangeable Ca^{++} in bone. Therefore K_d , and hence the amount of Sr^{89,90} exchanged, will remain constant as long as the mole fraction of Ca and other exchangeable cations in bone remain unchanged. Since a gm. of bone contains about 1 mm of exchangeable calcium, tracer levels of Sr^{89,90}, i.e. $\sim 10^{-10}$ M, cannot affect K_d , regardless of the extent to which the tracer Sr is adsorbed. In the in vitro experiments of Falkenheim et al. (19), 50 mg. of bone ash were equilibrated with 25 ml. volumes of solution-containing Ca++. Their bone sample presumably contained about 0.05 mm of exchangeable calcium. When the contacting solution contained less than 10^{-4} M, i.e. < 0.0025 mM of tracer alkaline earth, no appreciable change in K_d could be expected. However, a solution 10^{-3} M, equivalent to 0.025 mm, if adsorbed to the same extent as tracer levels, ~75 per cent or more as found for Sr and Ca (19, 22), would markedly reduce the mm of Ca in bone, and hence a sharp drop in the per cent adsorption must take place at about 10⁻³ M, in agreement with the experiments on bone (18, 19).

The preceding calculations explain why the injection of 0.01 to 10 μ c. of carrier-free Sr^{89,90} in the experiments of Murray and Bloom (23) resulted in no change in the per cent uptake.² In some experiments of Copp and Greenberg (24) it was thought that the injection of non-radioactive "carrier" strontium might dilute and wash out radioactive strontium. In view of the large amount of exchangeable calcium in bone it can be calculated from equation (2) that it would require about 60 mg. of Sr in a 200 gm. rat to affect the uptake of Sr appreciably. This was, indeed, found to be the case, but inasmuch as this amount of Sr is lethal or near lethal it is of no practical value.

^{*}We are, of course, disregarding any effects due to the radiation itself.

Elements such as Th, Pu, and Zr, because of their chemical properties, are most probably retained by bone through an irreversible type of non-specific surface adsorption. Therefore, the uptake and elimination of these elements by bone, in analogy to similar inorganic systems (21), should be unaffected by changes in bone resulting from age, diet, or other ordinary metabolic changes. A fuller discussion of these points together with pertinent experimental data will be given in a subsequent paper.

SUMMARY

- 1. The distribution and daily excretion of tracer levels of injected thorium and strontium were studied in rats receiving zirconium or sodium citrate intraperitoneally ½ hour after the administration of the radio-elements ("early" treated) or 3 days later ("late" treated). One group of rats received no other treatment.
- 2. In contrast to previous studies on plutonium and yttrium, zirconium citrate had no specific effect on Th or Sr metabolism other than that associated with the citrate part of the molecule.
- 3. The early administration of zirconium or sodium citrate resulted in about a 3-fold increase in the urinary excretion of Th and a 2-fold increase in the urinary excretion of Sr during the following 24 hours. No effect on the fecal excretion was found. The Th concentration in the liver, mesenteric lymph node, pancreas, and spleen was markedly reduced as a result of early sodium citrate administration. No significant changes in the metabolism of Th or Sr were found in the late treated groups.
- 4. The deposition of Th per gm. was highest in the soft tissues, particularly in the liver, while Sr accumulated mainly in the skeleton.
- 5. An interpretation of the results in terms of ion exchange, surface adsorption, and complex ion formation was presented.

We wish to express our appreciation to Rosie Hunter for the mounting and counting of many of the numerous samples required by this work.

BIBLIOGRAPHY

- Copp, D. H., Axelrod, D. J., and Hamilton, J. G., Am. J. Roentgenol., 58, 10 (1947).
- 2. Hamilton, J. G., Rev. Modern Phys., 20, 718 (1948).
- 3. Schubert, J., Science, 105, 389 (1947).
- 4. Schubert, J., J. Lab. and Clin. Med., 34, 313 (1949).
- Donnelly, G. L., Ross, C. J., Meroney, W. H., and Holman, R. L., Proc. Soc. Exp. Biol. and Med., 57, 75 (1944).
- 6. Hevesy, G., Radioactive indicators, New York (1948).
- 7. Kety, S. S., and Letonoff, T. V., Am. J. Med., 205, 406 (1943).
- 8. McClinton, L. T., and Schubert, J., J. Pharmacol. and Exp. Therap., 94, 15 (1948).

was able to overcome the action of the analogue competitively (cf. also Table II). If the amount of analogue needed when no pimelic acid was added is subtracted from the quantities necessary in the presence of 1 γ and of $3\frac{\pi}{4}\gamma$ of the metabolite, approximately 3 times as much additional analogue was required with 3 γ of pimelic acid as with 1 γ .

Antagonism between ϵ -(2,4-Dichlorosulfanilido)-caproic Acid and Biotin—When slightly more biotin than that which would cause stimulation of growth of B. tenuis cultured in test-tubes (see Table I) was added to the

Table II

Inhibition of Growth of B. tenuis Cultured in Flasks by \(\epsilon -(2,4-Dichlorosulfanilido) - \)

caproic Acid and Its Reversal by Pimelic Acid or Biotin

Analogue	Biotin	Pimelic acid	Turbidity*
γ per cc.	γ per cc.	γ per cc.	
0	0	0	70
20	0	0	60
. 50	0	0	82
100	0	0	98
100	0.0005	0	66
100	0	3	70

^{*} See foot-note to Table I.

TABLE III

Amounts of \(\epsilon -(2,4-Dichlorosulfanilido) \)-caproic Acid Which Caused Half Maximal Inhibition of Growth of B. tenuis in Presence of Various Quantities of Pimelic Acid

Pimelic acid added	Analogue needed for half maximal inhibition γ per cc .			
γ per cc.				
0	58			
1	85			
3	150			

basal medium, then quantities of the analogue which previously had completely suppressed growth no longer caused any inhibition. Under these conditions of biotin addition no effect of the analogue was noted until about 2 mg. per cc. were present. With such large concentrations of the analogue (i.e. above 2 mg. per cc.) neither biotin nor pimelic acid would overcome the inhibition of growth. At this high concentration the analogue probably shows a toxicity unrelated to its antimetabolite effects. Sulfonamide drugs in high concentration likewise have been found to behave similarly in that p-aminobenzoic acid will not nullify their action (2).

The antagonism between the sulfanilide and biotin would thus appear to be strictly non-competitive. Once the biotin which the bacillus would ordinarily elaborate from pimelic acid has been added from external sources, the inhibition caused by interference with this process is completely removed.

Correlation of Toxic Properties of ϵ -(2,4-Dichlorosulfanilido)-caproic Acid with Nutritional Requirements of Various Species for Biotin—A variety of microbial species was tested for inhibition of growth by the sulfanilido analogue in the manner described in a preceding section. The ability of

Table IV

Correlation of Toxicity of \(\epsilon \). (2,4-Dichlorosulfanilido)-caproic Acid with Nutritional Needs for Biotin

Organism		alogue n maximal	eed inl	ed for iibition	Nutritional requirement for biotin
		γ per	cc.		
Bacillus tenuis	58*				Not required, but stim- ulatory under certain conditions
Acetobacter suboxydans	350				Not required, but some- what stimulatory
Mycobacterium tuberculosis H37Rv	20†				Not required
Escherichia coli	No	effect	at	1000	" ""
" biotinless	"	"	"	1000	Required
Proteus strain 4	"	"	"	1000	•"
Staphylococcus aureus	"	"	"	300	"
Lactobacillus casei	"	"	"	500	"
" arabinosus	"	"	"	500	"
Leuconostoc mesenteroides	"	"	"	500	**
Hemolytic streptococcus H69D	"	"	"	500	**
Saccharomyces cerevisiae	"	"	"	1000	e c

^{*} When cultured in test-tubes, a value of 20 was uniformly found.

each species to grow in the basal medium free of biotin likewise was determined. The results are shown in Table IV. For species of which inhibition of growth occurred the action of the analogue was found to be overcome by biotin or by pimelic acid. Except for *E. coli*, the species which required biotin were not inhibited, and those which did not, were.

A fault in the argument seems to appear in these tests, because the presence of biotin in the medium is needed in order to test the biotin-requiring species for any effect of the analogue. We have just seen that biotin will nullify the effects of the analogue on the non-biotin-requiring species. In an effort to resolve this difficulty, the amount of biotin used in the tests

[†] Incubation time 4 days.

with the biotin-requiring forms was limited to less than the concentration necessary for maximal growth response. It was hoped that in this way any possible inhibition caused by the analogue might be unmasked. Some slight support for this hope was found with *B. tenuis* cultured in test-tubes in the presence of suboptimal amounts of biotin. Growth could be inhibited by the analogue but never completely. Instead, it was usually reduced to that obtainable with the suboptimal amount of biotin alone. Nevertheless, no adequate means of dealing with this fault in the experimental set-up has been found.

Effect of ϵ -(2,4-Dichlorosulfanilido)-caproic Acid on Biotin Synthesis by E. coli—A second fault in the argument appears from the finding that E. coli was not inhibited in growth by the analogue. Since this organism does not require biotin, one would expect it to be susceptible. In an effort to study this problem, the effect of the analogue on the synthesis of biotin by E. coli was examined. The bacteria were grown in the biotin-free basal medium for 2 days. Various amounts of the analogue ranging from 0 to 500γ -per cc. were added at the start of this period of growth. At the end, the cultures were heated at 100° for 5 minutes, centrifuged, and the supernatant solutions were analyzed for biotin according to the method of Eakin et al. (10). In the unsupplemented basal medium 0.008γ of biotin per cc. of culture was found, but no diminution in this amount was detected in the cultures grown in the presence of the greatest concentration of the analogue. Therefore, it seemed plain that the analogue had not inhibited the synthesis of biotin in this organism, just as it had not inhibited growth.

A plausible explanation of the failure of the analogue to inhibit the growth of E. coli might be that the organism forms sufficient pimelic acid to antagonize completely any practicable amount of the analogue. In the case of some sulfanilamide-resistant bacteria, the production of large amounts of p-aminobenzoic acid seems to account for their resistance to the drug (11). E. coli might owe its resistance towards the pimelic acid analogue to a similar ability to form sufficient pimelic acid. The biological assay of pimelic acid is complicated by the fact that biotin which might be present in the sample is far more potent in promoting growth of the organisms that require pimelic acid than is pimelic acid itself. An attempt was made to overcome this difficulty by destruction of the biotin. To this end a filtrate from a heated culture of E. coli grown in the biotin-free basal medium for 2 days was acidified to pH 2 and extracted five times with ethyl acetate. The material which was extracted was dissolved in water and treated with hydrogen peroxide to oxidize the biotin which it contained. The reaction mixture was then freed of peroxide with manganese dioxide, acidified, and extracted with ethyl acetate five times. The extracted material was evaporated and assayed for pimelic acid by making use of the stimulation of growth of B. tenuis which this acid produces when the organism is grown in test-tubes.² A very considerable stimulation was produced. However, the assay of pimelic acid as carried out with B. tenuis cannot be considered as conclusive because of uncertainties about its specificity. Therefore, the failure to explain adequately why the analogue does not inhibit E. coli must remain a serious fault in the argument about the basis of selectivity of action of this compound. Similar failures of the hypothesis as applied to the sulfonamide drugs could be enumerated, because some sulfanilamide-resistant streptococci are known which still do not require folic acid.

Toxicity of ϵ -(2,4-Dichlorosulfanilido)-caproic Acid for Mice—Since mice are believed to rely on an alimentary source of biotin, one might expect that the analogue of pimelic acid would not be harmful to these animals. To test this, young adult mice of the Rockefeller Institute strain were injected intraperitoneally with neutral solutions of the compound. Twelve mice so treated with single doses of 10 mg. of the analogue showed no toxic manifestations either immediately or after maintenance for 3 weeks on a stock diet.

Inhibition of Growth of B. tenuis by ϵ -Sulfonamidocaproic Acid and Its Dependence on Time of Incubation—When B. tenuis was grown in flasks for 20 hours, a marked inhibition was caused by this sulfonamide analogue of pimelic acid. Inhibition was complete with 100 γ of the analogue per cc. and half maximal effect was found with 20 γ per cc. Biotin or pimelic acid overcame these actions. However, when incubation was continued, growth began in the flasks containing the analogue, and after 2 days no retardation was found even with 500 γ per cc.

Stimulation of Growth by Subinhibitory Amounts of ϵ -(2,4-Dichlorosulfanilido)-caproic Acid—Concentrations of the analogue which were too small to cause inhibition of growth of B. tenuis produced stimulation. This effect was considerably greater when pimelic acid or biotin was added to the basal medium than in the absence of these addenda. The data of Table II will show the magnitude of the stimulation in the absence of these vitamins. Thus, like many other antimetabolites (12), this analogue of pimelic acid caused stimulation of growth when present in subinhibitory concentrations.

SUMMARY

 ϵ -(2,4-Dichlorosulfanilido)-caproic acid, an analogue of pimelic acid in which the dichlorosulfanilido group replaced one of the carboxyls of the metabolite, has been shown to inhibit the growth of several bacteria which

² It was also assayed for biotin and found to contain less than 1 per cent of that present in the original culture.

do not require biotin as a growth factor and not to affect those which do require biotin. One species, *E. coli*, was found which was insusceptible to the action of the analogue and yet did not require biotin. It thus deviated from the simple relationship. These facts were offered in support of a hypothesis to explain one of the bases of selectivity of action of antimetabolites. This postulate has been promulgated previously for the sulfon-amide drugs derived from *p*-aminobenzoic acid. According to it a selective agent can be produced by forming a structurally similar inhibitor of a *precursor* of a chosen metabolite. Such an analogue will then affect those species which can synthesize this metabolite, but will not harm those which cannot, and which rely on dietary sources of the chosen metabolite. The limitations of the experimental findings in relation to this hypothesis have been indicated.

BIBLIOGRAPHY

- 1. Woolley, D. W., Ann. Rev. Biochem., 16, 359 (1947).
- Nimmo-Smith, R. H., Lascalles, J., and Woods, D. D., Brit. J. Exp. Path., 29, 264 (1948).
- 3. Lampen, J. O., and Jones, M. J., J. Biol. Chem., 164, 485 (1946).
- 4. du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., Science, 96, 186 (1942).
- Marvel, C. S., MacCorquodale, D. W., Kendall, F. E., and Lazier, W. A., J. Am. Chem. Soc., 46, 2838 (1924).
- 6. Landy, M., and Dicken, D. M., J. Lab. and Clin. Med., 27, 1086 (1942).
- 7. Landy, M., and Dicken, D. M., J. Biol. Chem., 146, 109 (1942).
- 8. Woolley, D. W., and White, A. G. C., J. Exp. Med., 78, 489 (1943).
- 9. Dubos, R. J., and Middlebrook, G., Am. Rev. Tuber., 56, 334 (1947).
- 10. Eakin, R. E., Snell, E. E., and Williams, R. J., J. Biol. Chem., 136, 801 (1940).
- Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., Science, 97, 265 (1943).
- 12. Woolley, D. W., Physiol. Rev., 27, 308 (1947).

THE FORMATION OF 2,3-DIPHOSPHOGLYCERATE IN RABBIT ERYTHROCYTES: THE EXISTENCE OF A DIPHOSPHOGLYCERATE MUTASE

By S. RAPOPORT AND J. LUEBERING

(From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati)

(Received for publication, October 12, 1949)

The red blood cells of most mammalian species are distinguished by the presence of 2,3-diphosphoglyceric acid, which accounts for about half of their total acid-soluble P (1). The compound is stable in contrast to the labile 1,3-phosphoglyceric acid (2), which is considered an obligatory intermediate in the glycolytic cycle of all tissues. From studies with radioactive P it would appear that 2,3-diphosphoglycerate has a rapid turn-over comparable to that of other phosphate esters of the red cells (3), an indication that it lies either in the main path of their phosphate cycle or exists in free equilibrium with an obligatory intermediate. The mechanism of its formation is unknown. Similar uncertainty obtains with regard to the steps in its decomposition. In intact cells at a plasma pH below 7.35, breakdown occurs which is linked with maintenance of adenosine triphosphate (ATP) (4). In the unfortified hemolysate the compound is almost inert (5), but is decomposed readily when adenylic acid is added (6).

The present study is concerned with the mechanism of the formation of 2,3-phosphoglycerate (2,3-P-GA).

Methods

Most of the experiments were performed with hemolysates of rabbit erythrocytes, prepared by the addition of 2 volumes of distilled water to 1 volume of cells twice washed in the cold with 0.9 per cent NaCl. Hemolysates of beef cells were prepared in the same manner. Phosphate determinations were performed by the method of Fiske and Subbarow (7). Acid hydrolysis of the acid-soluble P was carried out for 15 or 150 minutes in N sulfuric acid in a boiling water bath. Total phosphoglycerate was determined by the naphthoresorcinol method (8) adapted to the photoelectric colorimeter, and 3-phosphoglycerate by polarimetry (9).

Results

Formation of Stable P in Rabbit Hemolysates—The formation of difficultly hydrolyzable P was studied in a system consisting of a hemolysate of rabbit erythrocytes containing iodoacetate and fluoride, incubated in the presence of ATP, 3-phosphoglycerate (3-P-GA), or both. The iodoacetate served to inhibit formation of phosphoglycerate by way of the phosphoglyceraldehyde dehydrogenase system, and the fluoride, decomposition of phosphoglycerate through enolase activity. In the amounts added, both inhibitors were highly effective. Table I presents the data of a representative experiment. It may be seen that some liberation of inorganic P occurred when ATP was added. The amount was greatest in Tube A, which contained both ATP and 3-P-GA. Disappearance of hydrolyzable P in significant amounts occurred only when both compounds were added.

Table I

Disappearance of Added Substrates from Rabbit Hemolysate

The incubation mixtures contained, per ml. of final volume, 0.5 ml. of a freshly prepared 1:3 water hemolysate of rabbit cells, 25 μ m of fluoride, and 20 μ m of iodoacetate. Where indicated in the table, 3 μ m of 3-P-GA and approximately 2 μ m of ATP were added. All additions were made in the form of sodium salts. The samples were incubated for 4 hours at 37°. The values are expressed as micromoles per 0.5 ml. of red cells.

			Pho	osphat	e analy	sis			
Experimental conditions	3	Inor	ganic		min. olysis		min. oly s is	3-P	-GA
		Total	Δ*	Total	Δ*	Total	Δ*	Total	Δ•
Tube O + ATP + 3-P-GA $^{\prime\prime}$ $^{\prime\prime}$ $^{\prime\prime}$ $^{\prime\prime}$ $^{\prime\prime}$ $^{\prime\prime}$	Initial Incubated	1.5 5.0	+3.6	14.7	1	18.4		10.0	-4.6
" B + "	"	4.4	+2.9	13.4	-1.3	17.5	-0.9	0.0	0.0
" C + 3-P-GA " D	"	1.4 1.6	-0.2	$\begin{vmatrix} 2.4 \\ 2.5 \end{vmatrix}$	-0.1	3.4 3.4	0.0	10.0 0.0	0.0

^{*} Differences of Tubes A and B versus O, and of Tube C versus D.

Independent determination of 3-P-GA by the polarimetric method, listed in the last column of Table I, indicates the disappearance of an amount of 3-P-GA equivalent to that of labile P transferred to the stable form.

These data would indicate the formation of 2,3-diphosphoglycerate from 3-P-GA and ATP, in consonance with an over-all reaction:

(1)
$$3-P-GA + ATP = 2, 3-P-GA + ADP$$

Although a one-step transphosphorylation according to Reaction 1 above is a possibility, it would appear more likely that the reaction proceeds in two steps as follows:

(2, a)
$$1,3-P-GA \rightarrow 3-P-GA + inorganic P$$

(3) 1,3-P-GA ≠ 2,3-P-GA (diphosphoglycerate mutase)

Reaction 2, because of the lability of 1,3-P-GA, should lead to an increase of inorganic P without change in the sum of inorganic and hydrolyzable P. The occurrence of Reaction 3, catalyzed by the mutase in question, would be indicated by the transfer of P from the hydrolyzable to the stable form. The occurrence of the Bücher reaction in rabbit hemolysates is suggested by the accelerated liberation of inorganic P when both ATP and 3-P-GA are present.

In Fig. 1 is presented the time curve of stable P formation, and in Figs. 2 and 3 the relation of the synthesis to the concentration of ATP and

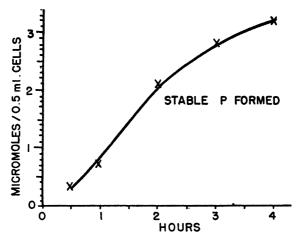


Fig. 1. Time curve of formation of stable P in rabbit hemolysate. The experimental conditions were the same as those described in Table I, except for the variation in incubation time.

3-P-GA. In Fig. 4 the pH-activity curve is portrayed, together with one presenting the sum of inorganic and stable P formed, both derived from ATP. It may be seen that formation of stable P has a pH optimum between 6.9 and 7.5, with rapid loss of activity on either side. The total amount of P transferred from ATP to either inorganic or stable form has a similar optimum, but a somewhat less steep fall of activity, particularly on the acid side.

In Table II are presented data on the effect of dilution of the hemolysate on the formation of stable P. It may be seen that, while the liberation of inorganic P reached a plateau and showed little change on further dilution, the formation of stable P declined. Such behavior may be expected in a system of stepwise reactions controlled by two or more enzymes. Apparently the rate of 2,3-P-GA formation decreased greatly with falling concentrations of 1,3-P-GA and fell below the rate of the spontaneous decomposition of the labile compound.

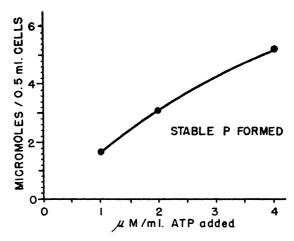


Fig. 2. The effect of ATP. The conditions were the same as previously described, except for the variation in the amount of ATP added.

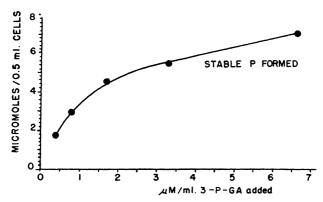


Fig. 3. The effect of 3-P-GA. The conditions were the same as before, except for the variation in 3-P-GA.

Beef Cells and Muscle—It appeared of interest to study beef cells which do not contain diphosphoglycerate. Such cells, like those of other tissues, are known to form 3-P-GA when incubated with fluoride and pyruvate, rather than diphosphoglycerate, which is formed by rabbit erythrocytes which contain diphosphoglycerate (11). Presumably they lack the enzyme necessary for the formation of 2,3-P-GA. In Table III is shown a

- 9. Gruber, C. M., and Halbeisen, W. A., J. Pharmacol. and Exp. Therap., 94, 65 (1948).
- 10. Seaborg, G. T., Rev. Modern Phys., 20, 585 (1948).
- Norris, W. P., and Kisieleski, W. E., Cold Spring Harbor Symposia Quant. Biol., 13, 164 (1948).
- Jones, D. C., and Copp, D. H., AECD-1962, Technical information division, Oak Ridge directed operations, United States Atomic Energy Commission.
- 13. Sollman, T., and Brown, E. D., Am. J. Physiol., 18, 426 (1907).
- 14. Haven, F. L., and Randall, C., J. Biol. Chem., 175, 737 (1948).
- Muntz, J. A., and Barron, E. S. G., Report CH-3708, Metallurgical Laboratory, University of Chicago (1947).
- 16. Kety, S. S., J. Biol. Chem., 142, 181 (1942).
- 17. MacKay, E. M., Carne, H. O., and Wick, A. N., J. Biol. Chem., 133, 59 (1940).
- Falkenheim, M., Neuman, W. F., and Hodge H. C., J. Biol. Chem., 169, 713 (1947).
- Falkenheim, M., Underwood, E. E., and Hodge, H. C., UR-53, University of Rochester, Atomic Energy Project, U. S. Atomic Energy Commission, Rochester, New York.
- Neuman, W. F., Neuman, M. W., Main, E. R., and Mulryan, B. J., J. Biol. Chem., 179, 325, 335, 341 (1949).
- 21. Schubert, J., Ion exchange; Theory and application, New York, 167 (1949).
- 22. Hodge, H. C., Gavett, E., and Thomas, I., J. Biol. Chem., 163, 1 (1946).
- Murray, R. G., and Bloom, W., Histopathology of irradiation, New York, 99 (1948).
- 24. Copp, D. H., and Greenberg, D. M., MDDC-1001, Technical information division, Oak Ridge directed operations, U. S. Atomic Energy Commission.

GROUPS INVOLVED IN THE ZIMMERMANN AND KOBER REACTIONS

By H. W. MARLOW

(From the Department of Biochemistry, Southwestern Medical College of the University of Texas, Dallas)

(Received for publication, July 18, 1949)

Colorimetric methods for the quantitative determination of many compounds have been developed, based on their specific chemical or physicochemical properties. Such tests include the Zimmermann (1) reaction for androgenic assay and the Kober (2) reaction for estrogenic assay. Modifications of each of these have been reported by Callow et al. (3). Cohen and Bates (4), Szego and Samuels (5), and Holtorff and Koch (6). Many of these modifications are for the determination of estrogens and androgens obtained from the extract of urine, feces, and body tissues. The value of these variations lies in the fact that certain groups inherent in the perhydro-1, 2-cyclopentanophenanthrene nucleus or its derivatives are involved. The color development in the Zimmermann reaction (Zimmermann (1) and others) depends upon a carbonyl group adjacent to a methylene carbon (-CH₂-CO-). For this reason many compounds not related to 17-ketosteroids will give positive results. Callow (3) has implied that the group -CH₂--CO--CH₂-- found in cyclopentanone is necessarily the one found in perhydro-1, 2-cyclopentanophenanthrene compounds giving a positive Zimmermann. If this were true, only estrone-16 would give a positive reaction. These data do not confirm this.

Marrian (7) has postulated that 16-ketoestrone is the basis for the red color developed in the Kober reaction, and that only α -diketones could give a positive Kober test. This was before 16-ketoestrone was synthesized and tested. Marlow (8) showed that little or no color is developed when 16-ketoestrone is treated with the Kober or Zimmermann reagent as compared to other estrogens.

EXPERIMENTAL

The compounds used in these experiments are pure crystalline compounds of proved structure. The quantity treated with the Zimmermann reagent has an arbitrary concentration of 72 γ , while that treated with the Kober reagents has 30 γ . Some of these compounds (estrone-16, 16-ketoestrone, etc.) have not been reported as subjected to the Kober and Zimmermann tests, since they have only been synthesized and their structures established very recently. These compounds are critical ones struc-

turally for the determination of groups involved in the color development of the Kober and Zimmermann reagents. The other compounds have been reported but are included for comparative effects.

The methods involved necessitated only minor modifications in those cited in references above. For clarification they are briefly outlined below.

Zimmermann—0.2 ml. of solutions (0.2 ml. of redistilled 95 per cent alcohol as blank) containing 72 γ was treated with 0.2 ml. of 5.5 n KOH and 0.2 ml. of 2 per cent *m*-dinitrobenzene in alcohol. The tubes were stoppered and allowed to stand for 45 minutes. They were then diluted with 10 ml. of 70 per cent alcohol solution.

Kober—1.0 ml. of an alcohol solution (1 ml. of redistilled alcohol as blank) containing 30 γ of the substance to be examined was evaporated to dryness in a hot water bath. 0.6 ml. of a fresh, modified Kober reagent (0.625 gm. of β-naphthol dissolved in 25 ml. of concentrated H₂SO₄) was added. The tubes were heated for 2 minutes (shaking at 45 and 90 second intervals) in a boiling water bath and then cooled in ice water for a few seconds. 0.6 ml. of acidulated water was added with agitation while the tubes were in the ice water. They were then reheated in boiling water for $1\frac{1}{2}$ minutes, being shaken well at 45 seconds. The tubes were transferred to an ice water bath for 10 seconds. Then 1.8 ml. of 65 per cent H₂SO₄ (1:1) were added. The solutions were mixed well and then transmission was determined in the Cenco-Sheard spectrophotelometer.

Spectrophotelometer—The instrument was set to give 100 per cent transmission at 508 m μ . (This is the peak exhibited by most of the compounds studied.) When the sliding exit slit was 5 mm., the entrance slit for light was generally set at about 0.8 mm. The transmission generally was measured from 400 to 550 m μ to establish the transmission curve over the significant part of the visible spectrum. Since several samples were read successively (a special sliding mechanism was employed which will be described in a later paper), the transmissions of the blanks were reset at the original figures to correct for any drift in the machine due to line voltage variations. In this way comparative successive readings could be made. The K value recorded was evaluated from the equation $K = \log I_o/I$, which is numerically equivalent to the optical density calculated from $D = 2 - \log_{10} T$, or $D = 2 - \log I_o/I$. All derivatives were equilibrated to be equivalent to a basic compound.

Zimmermann Test For Ketosteroids

It will be seen in Table I that there is some change in the transmission of light, or shift of the peak, when an OH group, or other groups (as methoxy, benzyloxy), is at position 3. For instance, 3-methyoxyestrone, 3-benzyloxyestrone, and estrone gave K values in that descending order.

Androsterone acetate is about as efficacious as those mentioned above, indicating neither interference of the acetate radical nor need of unsaturation in Ring A for highest color development. In all these cases the carbonyl group is at position 17, and a methylene carbon is at position 16. Estrone-

Table I
Structural Configuration of Steroids and Zimmermann and Kober Reagents

Compound	Position occupied	Group at position	Zimmermann reaction*	Kober reaction*
Estrone (1, 3)†	17	Carbonyl	0.278	1.025
Benzyloxyestrone (1, 3)	17	"	0.303	0.871
Methoxyestrone (1,3)	17	"	0.382	1.115
Androsterone acetate (1,				
3)	17	"	0.326	No peak
Equilenin (1, 3)	17	"	0.349	0.807 (490)
Equilin (1, 3)	17	"	0.304	0.494 (470)
Estrone-16 (2, 4)	16	"	No peak	No peak
Methoxyestrone-16 (2,				•
4)	16	4.6		" "
Benzyloxyestrone-16 (2,				
4)	16			<i>"</i>
Estriol (1, 2)	16, 17	ОН		0.479 (464); 0.498
Isoestriol-A (1, 2)	16, 17	"	"	0.695
α-Estradiol (1, 3)	17			0.532 (436); 0.497
β-Estradiol benzoate (1,				(, ,
3)	17	17 OH		0.497
3-Methoxy-16-keto-α-				
estradiol (1, 2)	16, 17	16 O, 17 OH	" "	1.056
16-Ketoestrone (1, 2).	16, 17	Carbonyl		No peak
3-Methoxy-16-ketoes-				
trone (1, 2)	16, 17	"		"
Testosterone (1, 3)	17	17 OH		0.334 (490)
Cholesterol (1, 3)	17	R		No peak
Doisynolic acid	Ring D out			" "
Marrianolic acid	" " "			"

^{*} Peak at 508 mµ unless otherwise indicated in parentheses.

16 differs in that a carbonyl group is now at position 16, with methylene carbons at both positions 15 and 17. No color is obtained with this compound and the Zimmermann reagents. 16-Ketoestrone, with carbonyl groups at both positions 16 and 17 and an adjacent methylene carbon in position 15, develops no color. When an OH or an R group is at position 17 and methylene carbons at both the 15 and 16 positions, no color de-

[†] Meaning of figures in parentheses in this column as follows: (1) 13 tert-carbon; (2) 15 CH₂; (3) 16 CH₂; (4) 17 CH₂.

velops. If Ring D has been broken, as in doisynolic acid and marrianolic acid, no color develops.

Kober Reaction

The above modified Kober reaction was employed with β -naphthol in concentrated H₂SO₄. Table I shows the peak values at 508 m μ as in the Zimmermann reaction. Here again, when the carbonyl group is at position 17 and the methylene carbon is at position 16, the greatest amount of color is produced. However, 3-methoxy-16-keto- α -estradiol is an exception to this. When carbonyl groups are at positions 16 and 17 and a methylene carbon is at position 15 (conditions under which the Zimmermann reaction gives no color), from 10 to 20 per cent absorption of light is noted, but no definite peak is observed.

Results

From a study of the groups affecting the intensity of color development in the Zimmermann and Kober reactions, the following results are reported.

Zimmermann Reaction—(1) Maximum color is developed when the compound contains a carbonyl group at position 17 and a methylene carbon adjacent at position 16 in the perhydro-1, 2-cyclopentanophenanthrene compounds. (2) When a carbonyl group is at position 16 and the two adjacent carbons are methylenic, or when there are carbonyl groups at both positions 16 and 17 and a methylenic carbon at position 15, no color is produced. (3) When an OH or R group is at position 17 with an adjacent methylenic carbon at position 16, no color development is observed. (4) If position 3 has a methoxy or benzyloxy group on it, there is a significant effect on the transmission of light. (5) Unsaturation of Ring A or Rings A and B does not significantly affect the color development.

Modified Kober Reaction—(1) Maximum color is developed when the compound contains a carbonyl group at position 17 and a methylenic carbon at the 16th position of all the estrogens tested. (2) If an OH group is at position 17, or at both positions 16 and 17, together with adjacent methylenic carbons, the K value is roughly 50 per cent of that of compounds with a carbonyl group at the 17th position. (3) Methoxyestrone gives more color but the benzyloxy derivative gives less color than estrone in the Kober test. (4) Carbonyl groups at position 16 or at both the 16th and 17th positions gave no color and hence showed no peak value. (5) A hydroxyl group at position 17 and a carbonyl group at position 16 show as much transmission activity as a carbonyl at position 17 with an adjacent methylenic carbon.

SUMMARY

- 1. In the Zimmermann reaction, maximum color development depends on a carbonyl group being at position 17 and a methylene carbon at 16. No other group on or modification of Ring D of the perhydro-1, 2-cyclopentanophenanthrene nucleus gives a positive value.
- 2. In the modified Kober reaction, maximum color development is noted as in the Zimmermann reaction above. Other groups in other positions give definite color development. Especially noteworthy is 3-methoxy-16-keto- α -estradiol which replaces the methylenic carbon at position 16 with a carbonyl group. Equilenin, which has an aromatic Ring B, gives a very high peak. Equilin, which is less unsaturated than equilenin, has a K value of approximately one-half that of equilenin.

The author wishes to acknowledge his indebtedness to the following for their contributions to this paper by generously furnishing pure samples for the tests: Research Division, the Schering Corporation, Bloomfield, New Jersey, for α -estradiol, β -estradiol benzoate, testosterone, and androsterone acetate; Research Laboratories, Parke, Davis and Company, Detroit, Michigan, for equilenin, theelin, and theelol; Dr. Ernest J. Umberger, Pharmacologist, Food and Drug Administration, Washington 25, D. C., for equilin; and Dr. Max N. Huffman and coworkers for benzyloxy- and methoxyestrone, 16-ketoestrone, 3-methoxy-16-keto- α -estradiol, estrone-16, isoestriol-A, doisynolic and marrianolic acids. The author also wishes to express his gratitude for the help of his technicians, Mrs. Tom Morgan and Mrs. John Little, for their contribution to the paper.

BIBLIOGRAPHY

- 1. Zimmermann, W., Z. physiol. Chem., 233, 257 (1935).
- 2. Kober, S., Biochem. J., 32, 357 (1938).
- 3. Callow, N., Callow, R., and Emmens, C. W., Biochem. J., 32, 1312 (1938).
- 4. Cohen, H., and Bates, R. W., J. Clin. Endocrinol., 7, 701 (1947).
- 5. Szego, C. M., and Samuels, L. T., J. Biol. Chem., 151, 587 (1943).
- 6. Holtorff, A. F., and Koch, F. C., J. Biol. Chem., 135, 377 (1940).
- 7. Marrian, G. F., Harvey Lectures, 34, 37 (1938-39).
- 8. Marlow, H. W., Endocrinology, 42, 479 (1948).
- 9. Umberger, E. J., and Curtis, J. M., J. Biol. Chem., 178, 275 (1949).

THE EFFECTS OF FEEDING EXCESS pl-METHIONINE AND CHOLINE CHLORIDE TO RATS ON A CASEIN DIET*

By JAY S. ROTH AND JAMES B. ALLISON

(From the Bureau of Biological Research, Rutgers University, New Brunswick)

(Received for publication, October 11, 1949)

It has been shown by du Vigneaud and coworkers (1-3) that the methyl group as it occurs in methionine, choline, and other sources is a dietary essential. These investigators and others have also shown (4-6) that man and other animals are capable of transferring this methyl group from one compound to another, as, for example, from choline to homocysteine to form methionine, and from methionine to guanidoacetic acid to form creatine. This process is known as transmethylation.

By the use of radioactive tracers (7) it has been proved that the methyl group of methionine may also be oxidized directly to carbon dioxide, as much as one-third of that ingested being oxidized by the rat in 52 hours.

The metabolism of methionine and choline also bears a close and important relationship to fat metabolism, since a lack of either of these dietary essentials will lead to the formation of fatty livers and hemorrhagic kidneys (8-9).

A new method of approach has been to feed excessive amounts of methionine, under which conditions certain striking physiological changes may be detected (10-11). It has been shown (12) with Sherman strain rats that the addition of 4.8 per cent methionine to a 12 per cent casein diet causes a marked loss in weight, a large part of which represents fat stores of the animal. The animals may continue to lose weight while in positive nitrogen balance under these conditions, which may be interpreted to mean that extensive catabolism of fat is continuing. At the same time there is a marked hypertrophy of the kidney.

If the effects observed are due to the increased demands of the body to metabolize methyl groups, either by oxidation or by elimination as the methyl group of creatinine, we might expect that methyl groups of choline, fed in excessive amounts, would tend to cause similar physiological changes. The work described below was designed to test the effects of feeding a high level of choline and to explain further the relationships existing between this compound and methionine.

^{*}This work was done under contract with the Office of Naval Research, Navy Department, Washington, D. C.

EXPERIMENTAL

Five groups of male, Sherman strain rats, weighing approximately 250 gm. each, were utilized. There were ten rats in each group, housed two in a cage. The basal diet consisted of the following ingredients per 1000 gm.: Labco casein 120 gm., sucrose 154 gm., dextrose 222 gm., dextrin 202 gm., lard 252 gm., Wesson's salt mixture (13) 17 gm., and agar 33 gm. To prepare the diet, 1400 gm. of water were utilized per 1000 gm. of solid materials. The agar was first dissolved in two-thirds of the boiling water, and then the lard was added, followed by a slurry of the other ingredients in the remainder of the water. Mechanical stirring was employed to obtain a nearly homogeneous mixture and any losses in water due to evaporation were made up. Just before the diet was ready to solidify, the following vitamin supplement, per kilo of dry diet, was stirred into the mixture: thiamine hydrochloride 2 mg.; pyridoxine hydrochloride 1.6 mg.; calcium pantothenate 40 mg.; niacin 40 mg.; 2-methyl-1,4-naphthoquinone 0.2 mg.: p-aminobenzoic acid 40 mg.: inositol 100 mg.: riboflavin 3.2 mg.: biotin 0.2 mg.; folic acid 0.2 mg.; cod liver oil, containing 1800 U.S.P. units of vitamin A and 180 U.S. P. units of vitamin D per gm., 10 gm.; and α -tocopherol 40 mg.

The five groups of rats received the following, in addition to the basic diet, per 1000 gm. of dry ingredients: Group I, 48 gm. (4.8 per cent) of premethionine plus 1 gm. of choline chloride; Group II, 70 gm. (7.0 per cent) of premethionine plus 1 gm. of choline chloride; Group III, 48 gm. (4.8 per cent) of premethionine (no choline chloride); Group IV, 48 gm. (4.8 per cent) of premethionine plus 13.5 gm. (1.35 per cent) of choline chloride; and Group V, 13.5 gm. (1.35 per cent) of choline chloride.

The animals in Group I served as controls. The other groups were fed the same weight of diet as was eaten by Group I. Collections of urine and feces were made as previously described (12). The nitrogen intake of Groups I, II, III, and V was approximately the same (Table I). Group IV had a higher intake owing to a higher nitrogen content of the diet. The diet containing 7 per cent DL-methionine (Group II) also had a higher nitrogen content, but the rats on this diet ate less than the controls. In Table II are listed the organ weights of the five groups.

Consideration of the data in Tables I and II shows the following.

The addition of 1.35 per cent choline chloride (Group V) caused no hypertrophy of the kidney, the values for this organ being identical with control values obtained from rats fed the basic diet alone (12). The animals fed excess choline chloride lost considerable weight during the 20 day experimental period, but this also occurs with the basic diet, owing to restriction of food intake. During the last 10 days of the experimental

period the animals fed excess choline chloride were in positive nitrogen balance and gained weight slowly. On the other hand, Sherman strain rats fed 4.8 per cent DL-methionine (Group I), although in positive nitro-

TABLE I

Effect of Dietary Supplements of DL-Methionine and Choline Chloride on Nitrogen Balance and Body Weight of Rats during 20 Days

There were five cages of animals in each group. Four 4 day collections were made from each cage and analyses performed in duplicate on each collection. The results were averaged to give the values (twenty values obtained on ten rats in each group) in the table.

		Supplement				Nitrogen	Weight	
Group No.	Methionine	Choline chloride	Ingested N	Urinary N	Fecal N	balance	change	
	per ceni	per cent	mg. per kg. per day	mg. per kg. per day	mg. per kg. per day	gm. per kg. per day	gm.	
I	4.8	0.1	538	423	82	+0.033	-36.6	
II#	7.0	0.1	515	668	74	-0.227	-67.0	
III	4.8	0.0	495	493	75	-0.073	-52.4	
IV	4.8	1.35	638	550	93	-0.005	-43.3	
V	0.0	1.35	515	400	106	+0.009	-33.6	

^{*} Sixteen values on eight rats.

TABLE II

Effect of Dictary Sources of DL-Methionine and Choline Chloride on Organ Weights of Rats

The values given are average wet weights per 100 gm. of body weight of ten animals in each group.

Group	Suppl	ement					.	Seminal
No.	Methio- nine	Choline chloride	Liver	Kidney	Adrenals	Thyroid	Testes	vesicles
	per ceni	per ceni	gm.	gm.	mg.	mg.	gm.	gm.
I	4.8	0.1	3.15	0.856	12.6	4.5	1.07	0.264
II*	7.0	0.1	2.89	0.900	10.8	4.0	1.15	0.257
III	4.8	0.0	3.04	0.902	14.4	5.1	1.19	0.157
IV	4.8	1.35	3.37	0.860	11.6	4.5	0.89	0.196
\mathbf{v}	0.0	1.35	2.87	0.636	11.6	3.9	1.08	0.384

^{*} Average of eight rats.

gen balance during the last 10 days, continued to lose weight slowly. The loss represented fat stores, since the water content of the tissues examined did not change and there was a striking lack of fat in the animal carcass.

The absence of choline chloride in the diet containing 4.8 per cent premethionine (Group III) aggravated the effects of the methionine. There was a greater loss in weight, a change from a slightly positive to a negative nitrogen balance, and a greater hypertrophy of the kidney. Lack of choline chloride also gave rise to atrophy of the seminal vesicles.

The addition of 1.35 per cent choline chloride to a diet containing 4.8 per cent pl-methionine (Group IV) did not significantly alter the results obtained by feeding 4.8 per cent pl-methionine alone (Group I).

A diet containing 7 per cent DL-methionine (Group II) caused extensive tearing down of the body tissues of the animals, the high negative nitrogen balance continuing at the same levels throughout the 20 day experimental period. Despite this breakdown of body tissue there was a marked hypertrophy of the kidney. There was also an increased excretion of creatine and creatinine under these conditions (Table III).

TABLE III

Creatinine and Creatine Excretion of Rats Fed Excess pl-Methionine

Average excretion for a 20 day period.

Excess methior ine fed	Choline fed	Creatinine	Creatine
per cent	per cent	mg per kg per day	mg per kg per day
7 0	0 1	39 2	6 3
4-8	0.1	28 9	9 4 (12)*
0 0 (Basic diet)	0.1	25 8	5.7 (12)

^{*} The numbers in parentheses refer to bibliographic references

These last results confirm the reports of Brown and Allison (11) who used rats of the Long-Evans strain. Strain differences are illustrated since the same effects can be obtained in Long-Evans rats with a lower methionine content of the diet. Differences in strains in regard to methionine uptake have also been reported by Rutman and coworkers (14).

DISCUSSION

Excessive quantities of methyl groups, fed in the form of choline chloride, can apparently be metabolized without causing any profound physiological changes in rats. The effects observed when excess premethionine is fed may be due, therefore, to the homocysteine portion of the molecule. Du Vigneaud has obtained evidence for this conclusion. The possibility also remains that the intact molecule may be necessary to bring about the observed changes. Hogan (15) has noted that homocystine and choline,

¹du Vigneaud, V., private communication.

when fed together to growing rats, retarded growth to an extent essentially the same as did an equimolar quantity of methionine, but when fed separately they repressed growth only slightly.

Since it has been demonstrated that glycine will, in part, counteract the effects of excess methionine (12, 15), these effects may be the result of excessive demands of the body for extra glycine, or more likely extra serine which may be formed in vivo from glycine (16). This is in agreement with the view that one pathway for the metabolism of homocysteine is its conversion to cysteine by combination of homocysteine with serine to form cystathionine (17–19). When the demand for serine may be very high, as when feeding 7 per cent DL-methionine, actual tearing down of body tissues may take place to meet the requirements, this tearing down of tissue causing an increased creatinine excretion. Significantly, the excretion of creatine is not materially higher when excess methionine is fed up to the level of 4.8 per cent in Sherman strain rats, even when extra glycine and arginine are fed in addition (12), conditions which should be expected to favor the formation of creatinine.

The relationship of the metabolism of methionine to fat metabolism remains obscure, but the continued catabolism of fat and loss of fat stores observed in animals in nitrogen balance on a diet containing 4.8 per cent added methionine would seem to indicate that the catabolism of methionine or homocysteine requires the simultaneous catabolism of fat. The report (20) that large doses of methionine fed to humans gave rise to acetone and β -hydroxybutyric acid ketosis lends credence to this conclusion.

SUMMARY

- 1. A level of 1.35 per cent choline chloride in a diet containing 12 per cent casein is well tolerated by rats of the Sherman strain, and no unusual physiological changes are observed, as occur when excess methyl groups are fed in the form of methionine.
- 2. The absence of choline chloride in a diet containing 4.8 per cent premethionine aggravates the effects of the excess methionine. In addition, atrophy of the seminal vesicles occurs.
- 3. A level of 7 per cent primethionine fed to Sherman strain rats causes extensive tearing down of body tissue, loss in weight, and increase in the excretion of creatinine. A marked hypertrophy of the kidney is also noted.

BIBLIOGRAPHY

- 1. du Vigneaud, V., Dyer, H. M., and Kies, M. W., J. Biol. Chem., 130, 325 (1939).
- du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., Proc. Am. Soc. Biol. Chem., J. Biol. Chem., 128, p. cviii (1939).

- du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., J. Biol. Chem., 131, 57 (1939).
- du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., J. Biol. Chem., 134, 787 (1940).
- du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., J. Biol. Chem., 140, 625 (1941).
- Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., J. Biol. Chem., 149, 519 (1943).
- Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., Melville, D. B., and du Vigneaud, V., J. Biol. Chem., 169, 757 (1947).
- 8. Best, C. H., and Huntsman, M. E., J. Physiol., 75, 56 (1932).
- 9. Griffith, W. H., and Wade, N. J., J. Biol. Chem., 131, 567 (1939).
- 10. Brown, J. H., Proc. Soc. Exp. Biol. and Med., 71, 612 (1949).
- 11. Brown, J. H., and Allison, J. B., Proc. Soc. Exp. Biol., and Med., 69, 196 (1948).
- 12. Roth, J. S., and Allison, J. B., Proc. Soc. Exp. Biol. and Med., 70, 327 (1949).
- 13. Wesson, L. G., Science, 75, 339 (1932).
- 14. Rutman, R., Dempster, E., and Tarver, H., J. Biol. Chem., 177, 491 (1949).
- 15. Hogan, J. M., Thesis, Rutgers University (1949).
- 16. Sakami, W., J. Biol. Chem., 178, 519 (1949).
- Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., J. Biol. Chem., 143, 559 (1942).
- 18. Stetten, D., Jr., J. Biol. Chem., 144, 501 (1942).
- 19. Binkley, F., and du Vigneaud, V., J. Biol. Chem., 144, 507 (1942).
- 20. Barclay, J. A., Kenney, R. G., and Cooke, W. T., Brit. Med. J., 2, 298 (1945.)

ENZYMATIC SYNTHESIS OF ORNITHURIC ACIDS*

BY R. W. McGILVERY AND PHILIP P. COHEN

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

(Received for publication, October 15, 1949)

Following the demonstration of the formation of p-aminohippuric acid (PAH)¹ from PAB and glycine (1-3) by rat liver, a parallel study of the formation of the p-aminoornithuric acids by chicken tissues was undertaken. This was done in order to compare the benzoylation of an optically active amino acid with that of glycine, and with the expectation the system might prove to be free of some of the difficulties besetting the study of PAH synthesis; namely, the low order of anaerobic activity when ATP is the sole source of energy for the reaction, and the apparent² inability to disrupt the cell particulates without destroying their activity in the synthesis.

In order to separate the benzoylations of the α - and δ -amino groups of ornithine, the α - and δ -monobenzoyl derivatives of L-ornithine were synthesized. These compounds, containing one free amino group, were then used as substrates, together with PAB, to produce enzymatically, as illustrated in Reactions 1 and 2, p-aminoornithuric acid or (presumably) p'-aminoornithuric acid which could be detected by the analytical method previously employed for PAH.

(1)
$$NH_2$$
— $(CH_2)_2$ — CH — NH — CO — $+$ $HOOC$ — NH_2 \longrightarrow

$$COOH$$
 α -Benzoyl-L-ornithine

PAB

 II_2N
— CO — NH — $(CH_2)_2$ — CH — NH — CO —

 $COOH$
 p' -Amino-L-ornithuric acid

^{*} Aided in part by a grant from the Wisconsin Alumni Research Foundation.

¹ The following abbreviations are used in this paper: PAH for p-aminohippuric acid, PAB for p-aminobenzoic acid, ATP and ADP for adenosine tri- and diphosphate, respectively.

² It has now been shown (R. W. McGilvery, unpublished data) that activity is present in extracts of acetone powders of rat liver and that the loss of activity on treating particulates with water is due in part to a diminished oxidative capacity and in part to an increased ATP-ase activity.

(2)
$$\sim$$
 CO-NH-(CH₂)₃-CH-NH₂ + HOOC \sim NH₂
COOH

5-Benzoyl-L-ornithine PAB

 \sim CO-NH-(CH₂)₃-CH-NH-CO-NH₂
 \sim NH₂

p-Amino-L-ornithuric acid

In exploratory experiments, it was found that chicken liver was devoid of activity toward either substrate, but that the kidney had a low activity toward both compounds. Attention was then concentrated on the α -benzoylation; i.e., on using δ -benzoyl-L-ornithine and PAB as substrates. It was found, as in the synthesis of PAH by rat tissue preparations, that the reaction would proceed anaerobically with ATP as the sole source of energy, and that the activity was associated with the insoluble cellular particulates. Only a survey of the properties of the system was made because of the small extent of the synthesis and because it was evident that further study would be less profitable than with the PAH system.

EXPERIMENTAL

Substrates—L-Ornithine hydrochloride (m.p. 232° (decomposition), Cl 21.08 per cent) was prepared from L-arginine by the method of Hunter (4).³ α-Benzoyl-L-ornithine (m.p. 225–226°, decomposition) was prepared via L-ornithuric acid by the method of Boon and Robson (5).

δ-Benzoyl-L-ornithine was prepared by a modification of the method of Neuberger and Sanger (6). 0.02 mole of L-ornithine hydrochloride was dissolved in 20 ml. of water and heated to boiling. Solid basic cupric carbonate was added in small portions until an undissolved excess remained. The excess was centrifuged in a heated tube and then triturated with 4 ml. of boiling water in the tube and recentrifuged. The combined clear, deep blue liquids were then chilled to 0° and 5 N NaOH added dropwise with stirring to about pH 11. The solution was then placed under brisk mechanical stirring in an ice bath and 10 ml. solutions containing 0.021 mole of benzoyl chloride in anhydrous toluene and 0.022 mole of NaOH in water were added in 1 ml. portions at 3 minute intervals. 5 minutes after the last addition, the solution was filtered and the solid, bright blue copper chelate of δ-benzoyl-L-ornithine obtained. The filtrate should have only a faint greenish blue tinge if benzoylation has been complete. The blue solid was thoroughly washed on the funnel, suspended in 70 ml. of water, and heated to boiling. H₂S then was passed into the

³ We are indebted to Dr. Santiago Grisolia for an arginase preparation.

boiling solution until no more blue particles could be seen. The CuS was filtered and thrice suspended in 15 ml. of boiling water, the suspension being each time resaturated with H₂S and filtered.

The combined filtrates were evaporated on the hot-plate under an air current to a volume of 20 to 30 ml., chilled, and filtered. The resultant δ -benzoyl-L-ornithine (yield 75 to 85 per cent) melted between 257° and 259° when dried. One recrystallization from water usually raised the melting point to 260–261° with small loss, and the melting point was constant with further treatment. Continued evaporation of the mother liquors enabled recovery of a small amount (about 5 per cent) of less pure material melting at 253–255°.

 δ -(p-Nitrobenzoyl)-L-ornithine (m.p. 241–242°, decomposition) was prepared in the same manner except that the chelate remained in solution and H_2S was passed directly into the reaction mixture at room temperature, and exhaustive washing of the CuS was not necessary. The yield was much lower (35 to 40 per cent).

p-Nitro-L-ornithuric (m.p. 188–189°, decomposition) and p'-nitro-L-ornithuric acids (m.p. 221–224°, decomposition) were prepared by conventional Schotten-Baumann reactions from the above two δ -substituted ornithine derivatives in good yield (85 to 95 per cent).

The corresponding amino compounds were obtained as white crystalline substances in fair to good yields by reduction with ammonium sulfide (7).

The amino compounds were converted to the corresponding p-(p-hydroxyphenyl)azo derivatives (1) for purposes of comparison. The melting points were as follows: L- α -hydroxy- δ -[p-(p-hydroxyphenyl)azobenzamido]-valeric acid, darkens at 262°, decomposes at 265°; p-(p-hydroxyphenyl)azo-L-ornithuric acid, darkens at 222°, decomposes at 224°; p'-(p-hydroxyphenyl)azo-L-ornithuric acid, darkens at 229°, decomposes at 230–231°. The optical density at 440 m μ of a 0.00001 m solution of each of these compounds was 0.399, 0.406, and 0.409 respectively. The absorption was measured in round cuvettes in the Coleman model 11 spectrophotometer. It is apparent that these derivatives have the expected equivalent weights within 3 per cent. (The agreement is more striking between the latter two compounds in which there is no question of the oxidation of the α -carbon atom by nitrous acid.)

As a further check on the purity of the series of compounds, the amino derivatives were analyzed by the method employed for PAH (1) and the absorption spectra determined in the Coleman instrument. These spectra are shown in Fig. 1 in comparison with that of the azo dye obtained under the same conditions from PAH.

Potassium fumarate was prepared by neutralization of a suspension of the solid acid and precipitation of the salt by stirring a mixture of the hot solution with several volumes of hot alcohol as it cooled. The resultant large clear crystals were washed with alcohol and air-dried, and gave an analysis for ash and water of hydration corresponding to $K_2C_4H_2O_4\cdot 2H_2O$ within 1.5 per cent for all preparations. This salt is more convenient than the free acid for the preparation of potassium fumarate solutions.

Other substrates have been described previously (1-3).

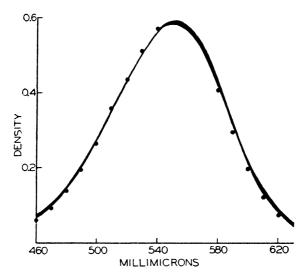


Fig. 1. Absorption spectra of ornithine derivative azo-coupled with N-(1-naphthyl)ethylenediamine. The spectra of the azo-coupled derivatives of δ -(p-aminobenzoyl)-L-ornithine and p-amino- and p'-amino-L-ornithuric acids lie within the black areas. Values obtained with PAH falling outside the area are shown as solid points. All were measured at 10 m μ intervals.

Analytical—The analysis was carried out as previously described (2). It was found that 62 per cent of the p-amino-L-ornithuric acid was not extracted under the conditions of the analysis. This low recovery introduced further error in the analytical method at low levels of synthesis, as may be seen in the spread of experimental points in the results.

Enzymatic Experiments—KCl homogenates and washed residues of chicken tissues were prepared and incubations were conducted as previously described (2, 3). The hydrosulfite-catalyst mixture was used in the center wells in all anaerobic experiments.

Results

Localization of Enzyme—Homogenates of chicken kidney and liver were tested for activity with both substrates, as were residues and supernatants of the kidney homogenate (Table I). The synthetic activity is localized

in the residue fraction. It has been noted previously (3, 8) that this residue contains the nuclei and a variable fraction of the mitochondria of the cell.

Stability of Enzyme—The activity of the entire homogenate toward either substrate is quite stable to storage in the cold (Fig. 2). The similarity of the rates of decline of the two activities might be interpreted as evidence

Table I Localization of Enzyme

Substrate concentrations, 0.001 m PAB, 0.015 m α - or δ -benzoyl-L-ornithine, 0.0025 m MgSO₄, 0.001 m ATP, 1 2 × 10⁻⁵ m cytochrome c, 0.0025 m potassium fumarate. Total volume 4.0 ml., made isotonic with potassium phosphate at pH 7.4. Incubation time, 1 hour. Residue prepared by centrifuging 10 minutes at 2000 × g without washing.

	Experiment 1					Experiment 2				
	α-Benzoylornithine 8-1		enzoylornithine δ-Benzoylornithine α-		α-Benzoylornithine		8-Benzoylornithine			
	Liver homo- genate	Kidney homo- genate	Liver homo- genate	Kidney homo- genate	Kidney residue	Kidney super- natant	Kidney residue	Kidney super- natant		
Tissue nitrogen, mg Total synthesis, μM	5.97 0.02	5 00 0 90	5.97 0.00	5.00 1.37	1.13 0.57	1.70 0.00	1.13 0.62	1.70 0.00		

that the same enzyme catalyzes both benzoylations. Actually, in this type of experiment, in which the activity is dependent upon the maintenance of ATP by aerobic phosphorylation, three events will influence the magnitude of the synthesis: changes in the activity of the oxidative systems, of the phosphatases attacking ATP, and of the synthesizing enzyme itself. Data obtained in another connection with the PAH systems lead to the conclusion that the loss of activity of the residue under various conditions, such as storage, water treatment, acetone drying, etc., is due primarily to an interference with the oxidative metabolism or to an increase in the phosphatase activity. This is probably the cause of the decline in the activity exhibited in Fig. 2 and no conclusions about enzyme identity can be drawn.

Substrate Concentration—As is shown in Fig. 3, a large excess of the ornithine derivatives is necessary to saturate the system, although this effect is not as pronounced as that with glycine in the PAH synthesis (2). It is possible that the high level of the amino acid acts to inhibit phosphatases as shown by Bodansky (9) and thereby aids in the maintenance of either ATP or phosphorylated cofactors necessary for the oxidation of fumarate. In a similar experiment it was demonstrated that 0.0008 M PAB saturated the system. At this point in the study, work with the α -benzoyl com-

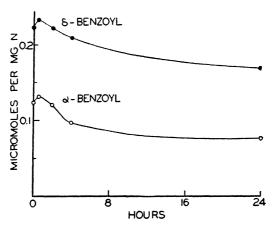


Fig. 2. Stability of KCl homogenate to storage in the cold. A KCl homogenate was placed at 0° and samples were withdrawn at intervals for enzymatic test. The concentrations and conditions were as in Table I. Tissue nitrogen, 4.84 mg. per flask.

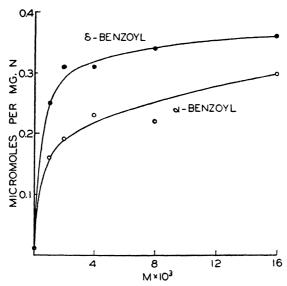


Fig. 3. Effect of α - and δ -benzoyl-L-ornithine concentration on the synthesis of the *p*-amino-L-ornithuric acids. The concentrations and conditions were as in Table I. Washed residue nitrogen, 1.07 mg. per flask.

pound was discontinued, since it showed less activity and the α -benzoylation was of primary interest.

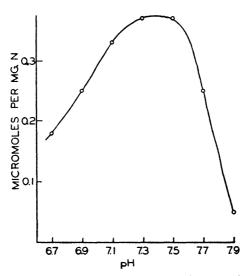


Fig. 4. Effect of pH on p-amino-L-ornithuric acid synthesis. The concentrations and conditions were as in Table I. Washed residue nitrogen, 3.20 mg. per flask.

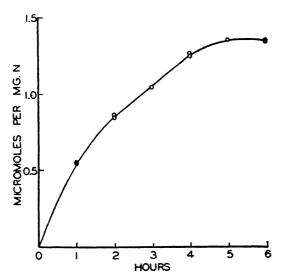


Fig. 5. Synthesis of p-amino-L-ornithuric acid as a function of time. The concentrations and conditions were as in Table I except that ATP was 0.002 m, potassium fumarate 0.01 m, and cytochrome c was eliminated. Washed residue nitrogen, 2.46 mg. per flask.

pH and Time Curves—The entire system exhibits a pH optimum at 7.4 (Fig. 4). Here again it is to be noted that this is an optimum for all of the enzymatic factors affecting the synthesis taken together and not for the synthesizing enzyme per se. The system continues synthesis for 5 hours under the conditions of the experiment (Fig. 5), finally achieving over 90 per cent coupling of the PAB available.

Miscellaneous Factors—In the experiments of Table II, it was established that calcium is inhibitory, as is generally the case in systems dependent upon aerobic phosphorylation. Magnesium is required for full activity,

TABLE II

Effect of Various Additions

Substrate concentrations, 0.001 m PAB, 0.015 m δ -benzoyl-L-ornithine. Total volume 4.0 ml., made isotonic with potassium phosphate at pH 7.4. Incubated 1 hour with air as gas phase. 1.61 mg. of washed residue nitrogen per flask.

Addition			Molarities			
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$.0038 0.00 .001 0.00 .0025 0.00	38 1 0.001	0.015 0.001 0.0025 1.2	0.0038 0.001 1.2	0.0038 0.001 0.01 1.2	0.0038 0.001 0.0025
Synthesis per. mg. N, µµ	.28 0.00	0.18	0.28	0.00	0.38	0.30

and $0.0015~\mathrm{m}$ is adequate to saturate the system. At least $0.01~\mathrm{m}$ fumarate is required for full aerobic activity and no synthesis could be detected in its absence. Unlike the rat liver residue, the chicken preparation does not require added cytochrome c for maximum aerobic synthesis.

ATP Requirement—The dependence of the reaction on the presence of ATP is clearly shown under aerobic and anaerobic conditions in Fig. 6. A higher saturation level is required than for the PAH systems (3) in which 0.0005 m ATP is adequate with aerobic conditions, under which its regeneration through oxidative phosphorylation is possible. The small extent of synthesis obtained anaerobically (Fig. 7) is explained by the rapid breakdown of ATP in this type of tissue preparation, and the increase due to higher ATP concentrations results largely from the longer time required to complete its destruction by the phosphatases.

Identification of Product—250 ml. of a medium containing 0.002 m ATP, 0.01 m potassium fumarate, 0.0025 m MgSO₄, 0.015 m δ-benzoyl-L-ornithine, 0.001 m PAB, and chicken kidney washed residue containing 156 mg. of nitrogen were incubated 6 hours at 38° in five 250 ml. Erlenmeyer

flasks with shaking. 10 gm. of solid trichloroacetic acid were added with stirring and the precipitated protein centrifuged. At this point a total of $42 \mu M$ of p-aminoornithuric acid was found in the clear centrifugate. The

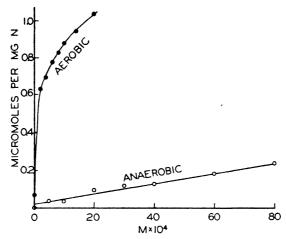


Fig. 6. Effect of ATP on aerobic and anaerobic synthesis of p-amino-L-ornithuric acid. The concentrations and conditions were as in Fig. 5, except that fumarate was omitted in the anaerobic experiment. Washed residue nitrogen, 2.13 mg. per flask.

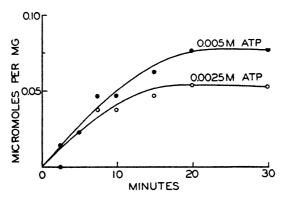


Fig. 7. Anaerobic synthesis of p-amino-L-ornithuric acid as a function of time. The concentrations and conditions were as in Table I, except that fumarate and cytochrome c were omitted and ATP concentrations were as given on the figure. Washed residue nitrogen, 2.18 mg. per flask.

solution was adjusted to pH 3.8 with solid sodium citrate and extracted three times with 3 volumes of ether and once with 1 volume of benzene. At this point, 27 µm of product and 0.0 µm of PAB remained. The solu-

tion was then diazotized, coupled with phenol, and carried through the sequence of ether and bicarbonate extractions as previously described (1). The final twice recrystallized yellow-orange product, 7.3 mg., 15.5 μ M, melted at 224° and gave a mixed melting point with p-(p-hydroxyphenyl)-azo-L-ornithuric acid of 224°. Solutions of the isolated product and known derivative containing 0.0137 and 0.0139 μ M per ml. in 0.1 N NaOH were made and the absorption spectra of the two solutions determined in the Beckman instrument with a 1 cm. light path. These spectra, reduced to

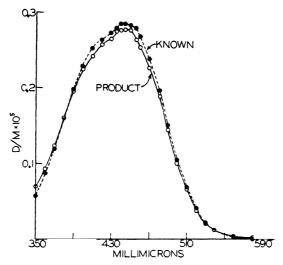


Fig. 8. Absorption spectra of p-(p-hydroxyphenyl)azo derivative of reaction product and known p-(p-hydroxyphenyl)azo-L-ornithuric acid in 0.1 n NaOH. The light path was 1 cm. The measurements were made in a Beckman spectrophotometer. Slit width 0.15 mm. to 410 m μ , 0.10 mm. to 430 m μ for the unknown, 435 m μ for the known, and 0.05 mm. above these wave-lengths.

the absorption of a 0.00001 m solution, are shown in Fig. 8. The anomaly in the curves near 430 m μ occurred owing to the necessity of changes in the slit width.

DISCUSSION

At the time the studies on benzoylation were begun in this laboratory, the formation in cell-free preparations of only one other amide, acetylsulf-anilamide, had been studied (10). While Lipmann had clearly enunciated the principle that high energy phosphate could be, and is, used in the synthesis of amides, uncertainty still existed in the minds of many investigators. In the brief ensuing period the dependence of not only PAH synthesis, but also the syntheses of such divergent amides as glutamine

(11), citrulline (12), glutathione (13), and now ornithuric acid, on the presence of ATP as an energy donor makes it certain that the view of Lipmann is correct and that the general formulation can be made:

$$R-NH_2 + HOOC-R' + ATP \rightarrow R-NH-CO-R' + ADP + H_2PO_4$$

While this expression represents the over-all reaction, some common intermediate must exist between the amide-forming reaction and the phosphate-hydrolyzing reaction. This intermediate could be, on theoretical grounds, any of the following possibilities (where E is the synthesizing enzyme): R—NH—PO₃H₂, R'—CO—OPO₃H₂, RNH—ADP, R'—CO—ADP, E—OPO₃H₂, E—ADP. Which of these possibilities is the correct one still remains unknown.

SUMMARY

- 1. The conjugation of PAB with α and δ -benzoyl-L-ornithine has been studied.
- 2. The synthesis is accomplished by chicken kidney washed residues, but not by preparations of chicken liver.
- 3. ATP will support the reaction anaerobically. Under aerobic conditions potassium fumarate addition markedly accelerates the reaction by permitting regeneration of hydrolyzed ATP.
- 4. p-Amino-L-ornithuric acid has been isolated as the p-(p-hydroxy-phenyl)azo derivative.

BIBLIOGRAPHY

- 1. Cohen, P. P., and McGilvery, R. W., J. Biol. Chem., 166, 261 (1946).
- 2. Cohen, P. P., and McGilvery, R. W., J. Biol. Chem., 169, 119 (1947).
- 3. Cohen, P. P., and McGilvery, R. W., J. Biol. Chem., 171, 121 (1947).
- 4. Hunter, A., Biochem. J., 33, 27 (1939).
- 5. Boon, W. R., and Robson, W., Biochem. J., 29, 2864 (1935).
- 6. Neuberger, A., and Sanger, F., Biochem. J., 37, 515 (1943); 38, 119 (1944).
- 7. Org. Syntheses, 2nd edition, coll. 1, 52 (1941).
- 8. Schneider, W. C., J. Biol. Chem., 176, 259 (1948).
- 9. Bodansky, O., J. Biol. Chem., 174, 465 (1948).
- 10. Lipmann, F., J. Biol. Chem., 160, 173 (1945).
- 11. Speck, J. F., J. Biol. Chem., 179, 1405 (1949).
- 12. Cohen, P. P., and Hayano, M., J. Biol. Chem., 172, 405 (1948).
- 13. Johnston, R. B., and Bloch, K., J. Biol. Chem., 179, 493 (1949).

THE EFFECT OF ETHIONINE ON TRANSMETHYLATION FROM METHIONINE TO CHOLINE AND CREATINE IN VIVO*

By SOFIA SIMMONDS, ELIZABETH B. KELLER, JOSEPH P. CHANDLER, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, October 20, 1949)

In order to determine the specificity of the methyl group of methionine in the conversion of methionine to cystine in vivo, Dyer (1) tested ethionine (S-ethylhomocysteine) to see whether it would replace cystine in the diet. She found that ethionine, in contrast to homocystine and methionine, would not support the growth of young rats on a diet devoid of cystine and containing suboptimal amounts of methionine. It was concluded, therefore, that deethylation of ethionine to yield homocysteine did not occur in the rat. Further evidence in support of this conclusion was presented by Shen and Lewis (2), who found that the rabbit excreted the sulfur of ethionine largely as extra neutral sulfur.

Dyer (1) also observed that supplementation of a 5 per cent casein diet with 0.54 per cent ethionine produced a marked toxicity in rats. The food consumption of animals on the ethionine-containing diet dropped to about two-thirds of that found with the unsupplemented 5 per cent casein diet; animals receiving ethionine lost weight and died after about 10 days, while animals on the unsupplemented diet neither gained nor lost weight. At autopsy, however, no gross pathology due to the toxic effects of ethionine was observed. The toxicity of ethionine could be overcome by including a molar equivalent of methionine in the diet. In this connection, it is of interest that ethionine has been found to inhibit the growth of Escherichia coli (3, 4) and Plasmodium knowlesi (5), and the growth inhibition for both of these organisms could be reversed by methionine. Although these data do not provide any clue to the mechanism of the action of ethionine, its behavior as a metabolic antagonist may be attributed to interference with some phase of the metabolism of methionine.

Farber, Simpson, and Tarver (6) have reported that ethionine apparently interferes with protein synthesis, since experiments both *in vivo* and *in vitro* showed that ethionine inhibited the incorporation into liver

^{*} The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

protein of S³⁵ from labeled methionine and C¹⁴ from labeled glycine. Experiments *in vivo* with both amino acids indicated that additional methionine would reverse the inhibition. These authors also observed that ethionine inhibited the conversion of methionine to cystine and that this inhibition, also, could be reversed by additional methionine. They suggested that ethionine probably prevented the demethylation of methionine. Stekol and Weiss (7) recently have shown that choline as well as methionine reverses the growth inhibition caused by ethionine in the rat.

The investigation described in the present paper was designed to determine whether ethionine interferes with the transmethylation reactions involved in the formation of choline and creatine. Methionine labeled with deuterium in the methyl group was administered to adult rats and, after a suitable period, the deuterium content of the body choline and creatine was determined as a measure of the extent of transmethylation. Two experiments were carried out, each with a group of four rats. In each experiment, two animals were given ethionine in addition to the labeled methionine, and two animals served as controls, receiving only labeled methionine. Since it was known that the amount of deuteriomethyl transferred from dietary methionine to tissue choline and creatine in a given time is dependent upon the amount of deuteriomethionine ingested (8), the food intake of each group of rats was controlled by the paired feeding technique.

Preliminary feeding experiments to determine the level at which ethionine may be incorporated in the diet without seriously reducing the food consumption of test animals showed that when a basal diet containing 0.7 per cent methionine was supplemented with 0.7 per cent ethionine the daily food intake dropped from over 10 gm. to 1 to 2 gm. per day in 3 days. However, when the ethionine supplement was reduced to 0.5 per cent, the daily food intake dropped only to 7 to 8 gm. The latter diet, in which the molar ratio of ethionine to methionine is about 2:3, was used for the feeding experiments with labeled methionine. In view of the fact that Farber et al. (6) have found that ethionine interferes with the conversion of methionine to cystine in vivo, it should be noted that the synthetic diet employed in our experiments contained 0.5 per cent cystine.

At the end of the feeding period, the rats were sacrificed, and choline and creatine were isolated from the tissues of each animal and analyzed for deuterium. The data showed that the presence of ethionine in the diet reduced the amount of choline synthesized from dietary methionine by about 20 per cent. On the other hand, the amount of creatine synthesized from dietary methionine was not decreased by the presence of ethionine in the diet.

EXPERIMENTAL

Feeding of DL-Ethionine and Deuterio-DL-methionine—The four adult male rats in each group were fed ad libitum a choline-free amino acid diet for a control period of 8 days, at the end of which time they had regained their initial weight. The percentage composition of this basal diet was as follows: amino acid mixture (9, 10) 21.3, L-cystine 0.5, DL-methionine 0.7, sucrose 53.5, hydrogenated vegetable oil 19, corn oil 1, and Osborne and Mendel's salt mixture (11) 4. The fat-soluble vitamins were included in this diet in the following amounts per 100 gm. of diet: 4000 U. S. P. units of vitamin A, 400 U. S. P. units of vitamin D, 1 mg. of α -tocopherol acetate, 0.1 mg. of 2-methyl-1,4-naphthoquinone. The B vitamins were given twice a day as a supplement. Each rat received daily 20 γ each of thiamine

Table I
Food Consumption and Weight Changes

	Control period			period	Experime	ental period	
Group No	Rat No	Food intake	Initial and final weight	Diet	Food intake	Final weight	
		gm per day	gm		gm per day	gm	
1	1	14.0	260-254	Ethionine	8.1	242	
	2	12.4	266-273	"	8.0	254	
	3	11.6	270-272	Control	8.0	252	
	4	12.9	250-247	"	8.1	232	
2	5	11 4	244-246	Ethionine	7.7	235	
	6	14.0	236-260	"	7.6	238	
	7	10.5	232-239	Control	7.6	230	
:	8	10 7	253-254	"	7.6	256	

hydrochloride, riboflavin, pyridoxine hydrochloride, and nicotinic acid, 0.2 mg. of calcium dl-pantothenate, and 25 mg. of choline-free ryzamin-B (9).

During the subsequent experimental period, two rats in each group were given 0.5 per cent pre-ethionine (1), which replaced an equivalent amount of sucrose in the basal diet, and all the rats received in place of the non-isotopic methionine 0.7 per cent deuterio-pre-methionine (12), containing 89.4 atom per cent deuterium in the methyl group.

During the experimental period, the food intake of all rats in each group was limited, by the paired feeding technique, to the amount eaten by the rat with the lowest food consumption. The food consumption and weight changes of the rats during both the control and experimental periods are given in Table I. It will be noted from the data in Table I that the presence of the ethionine in the diet decreased the food consumption from over 10 gm. per day to about 8 gm. per day. With Group 1 the experimental period lasted 9 days, and with Group 2, 14 days.

Creatine was isolated from the carcass of each rat as creatinine potassium picrate (12). Analysis of the recrystallized picrates for creatinine with the Jaffe color reaction indicated that they were pure within the limits of accuracy of the colorimetric method. Choline was isolated from each rat carcass as the choline chloroplatinate (12). The chloroplatinates from Group 1 were recrystallized several times from alcohol-water mixtures, and those from Group 2 were recrystallized three times from water. The platinum determinations for each of the isolated chloroplatinates agreed with the theoretical values.

The creatine and choline derivatives were analyzed for deuterium and the deuterium in the methyl group was calculated on the assumption that

Choline and	d Creatine Sy	inthesis in Presei	nce and Absence of L	netary Ethionine
Group No.	Rat No	Diet	Per cent of creatine methyl derived from dietary methionine*	Per cent of choline methyl derived from dietary methionine*
1	1	Ethionine	†	$7\ 20\ \pm\ 0.10$
	2	"	6.85 ± 0.18	8.66 ± 0.10
	3	Control	5.50 ± 0.18	10.5 ± 0.08
	4	"	5.68 ± 0.18	10.4 ± 0.07
2	5	Ethionine	$14\ 45\ \pm\ 0\ 31$	$14\ 65\ \pm\ 0\ 14$
	6	"	12.80 ± 0.36	$14\ 82\ \pm\ 0\ 16$
	7	Control	$12.89~\pm~0~36$	$18\ 81\ \pm\ 0\ 16$
	8	"	+ 12 44 $+$ 0 36	18.76 ± 0.16

TABLE II

Choline and Creatine Synthesis in Presence and Absence of Dietary Ethionine

all the deuterium in the compounds was present in the methyl group (12). The results are recorded in Table II.

SUMMARY

DL-Ethionine (S-ethyl-DL-homocysteine) was fed to adult male rats at the level of 0.5 per cent in a synthetic amino acid diet containing 0.7 per cent deuterio-DL-methionine. The deuteriomethyl content of the tissue choline and creatine was determined and compared with that of choline and creatine isolated from pair-fed controls which received labeled methionine but no ethionine. It was observed that ethionine decreased the amount of transmethylation from methionine to choline by about 20 per cent. The amount of creatine synthesized from dietary methionine was not decreased by the presence of ethionine in the diet.

^{*} Atom per cent deuterium in methyl groups of isolated compound
Atom per cent deuterium in methyl group of methionine fed

† Sample lost.

The authors wish to express their appreciation to Mrs. Josephine T. Marshall for the analyses reported in this paper and to Miss Miriam L. Thompson for her assistance with some of the isolations.

BIBLIOGRAPHY

- 1. Dyer, H. M., J. Biol. Chem., 124, 519 (1938).
- 2. Shen, C.-W., and Lewis, H. B., J. Biol. Chem., 165, 115 (1946).
- 3. Harris, J. S., and Kohn, H. I., J. Pharmacol. and Exp. Therap., 73, 383 (1941).
- Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., Jr., J. Am. Chem. Soc., 67, 290 (1945).
- 5. McKee, R. W., and Geiman, Q. M., Federation Proc., 7, 172 (1948).
- 6. Farber, E., Simpson, M., and Tarver, H., Federation Proc., 8, 196 (1949).
- 7. Stekol, J. A., and Weiss, K., J. Biol. Chem., 179, 1049 (1949).
- Cohn, M., Simmonds, S., Chandler, J. P., and du Vigneaud, V., J. Biol. Chem., 162, 343 (1946).
- du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., J. Biol. Chem., 131, 57 (1939).
- du Vigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., J. Biol. Chem.,
 155, 645 (1944).
- 11. Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 37, 572 (1919).
- du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., J. Biol. Chem., 140, 625 (1941).

THE PARTITION RATIO OF ALCOHOL BETWEEN AIR AND WATER, URINE AND BLOOD; ESTIMATION AND IDENTIFICATION OF ALCOHOL IN THESE LIQUIDS FROM ANALYSIS OF AIR EQUILIBRATED WITH THEM

By R. N. HARGER, B. B. RANEY, E. G. BRIDWELL, AND MARY F. KITCHEL (From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis)

(Received for publication, August 8, 1949)

Accurate information on the partition ratio of alcohol between air and water or body fluids is desirable for several reasons. The values for blood are of fundamental importance in analytical methods in which the alcohol concentration of the breath is employed to predict the level of blood alcohol. A comparison of the figures for blood and urine will shed light on the question whether the level of urinary alcohol represents simple diffusion or true secretion. As pointed out by us in 1939 (1), one may use the air-liquid ratios for water or urine to determine quickly the concentration of alcohol in these fluids from analysis of air equilibrated with them.

Published data for air-liquid partition ratios, covering concentrations of alcohol below about 10 per cent in water or body fluids and temperatures of 0-40°, are few and some of them are quite conflicting. together with our own results reported in this paper, are given in Figs. 5 and 6. The previously published determinations of air-liquid alcohol partition ratios include the following: water at 25° by Foote and Scholes (2), Thomas (3), and Dobson (4); water at 15-26° by Thomas; water at 39.76° by Wrewsky (5); blood at 31-40° by Liljestrand and Linde (6): water, urine, and blood at 10-40° by Haggard and Greenberg (7) in 1934: and water and blood at 20-40° by Haggard et al. (8) in 1941. The results for air-water at 25° obtained by Foote and Scholes, Thomas, and Dobson agree quite well, but the ratio for this temperature reported by Haggard and Greenberg is very much higher. Haggard and Greenberg's 1934 figure for air-water at 20° is 3 times that obtained by Thomas, and their 1934 results for air-blood are much higher than those reported by Liljestrand and Linde. With the odd exception of blood at 40°, the 1941 air-liquid figures of Haggard et al. are much lower than their 1934 results. However, most of the 1941 figures of Haggard et al. remain higher than those of other investiga-Haggard et al. ascribe the errors in their 1934 results as being due to loss of alcohol caused by the condensation of water in the cooler parts of their apparatus. This explanation lacks plausibility because moisture condensation would cause low, not high, results and also because their greatest errors were at room temperature at which such condensation would be negligible.

During 1929 two of us (B. B. R. and R. N. H.) made a comprehensive study of the air-water alcohol partition ratio for the range of 1–37°, employing alcohol concentrations from 1.0 to 20 mg. per cc. Our results agreed quite well with the few which then appeared in the literature. Shortly afterwards we studied the air-blood alcohol partition ratio. Since Haggard and Greenberg's 1934 paper gave very much higher air-l quid partition ratios, we conducted a large number of additional determinations, with equilibrium approached both from below and from above. These agreed with our earlier results. We made further tests following the publication of the 1941 paper by Haggard et al., but these results simply confirmed our earlier findings.

It should be possible to determine the concentration of alcohol in the water phase from analysis of the air phase, plus the air-water partition ratio for the temperature employed. Here W is the unknown in the equation $k_t = A/W$, where k_t is the Ostwald partition ratio (9) for the temperature used and A and W are the weights of alcohol per unit volume of air and water, respectively. Our experiments showed that this could be accomplished with a satisfactory degree of accuracy, and very rapidly. We found that urine may also be analyzed by this method, by use of the k_t for urine.

Finally, analysis of air equilibrated with the distillate from body materials may be used as confirmatory evidence that a reducing substance in this distillate is all alcohol. This use of partition ratio data is analogous to other physical measurements, such as specific gravity, index of refraction, etc.

EXPERIMENTAL

Partition Ratio of Alcohol for Air-Water, Air-Blood, and Air-Urine

Analytical Method for Alcohol—The alcohol content of both air and liquid phases was determined by the Harger dichromate method (10). Jetter (11) stated that the accuracy of this method is very satisfactory, and the titration procedure for excess dichromate has been employed in a number of other analytical methods for alcohol (12–17). We chose gas volumes and aliquots of solutions or distillates which would consume 70 to 90 per cent of the dichromate. The accuracy of factor W in our calculation formula (10) was also slightly improved by running the acid blank with 0.2 cc. of the dichromate solution and, after titrating, adding a second 0.2 cc. of the dichromate and retitrating. If the first and second titration figures are represented by a and b, respectively, then W = B + a - b. B is obtained by a third titration with 1 cc. of the dichromate following titration b. In drying the reaction tubes after they had been thoroughly

washed we avoided contact of the open ends with paper toweling or other substances which might permit dissolved organic matter to creep up inside the tube.

Three procedures were used for equilibrating air with the liquid containing alcohol.

Method 1—The apparatus for this procedure is of the conventional type and is shown in Fig. 1. Tubes 1 to 4 each contain 10 to 15 cc. of the alcohol solution being analyzed, and Tubes a and b each contain 8 cc. of 50 per cent (by volume) sulfuric acid for absorbing alcohol. At first we used a

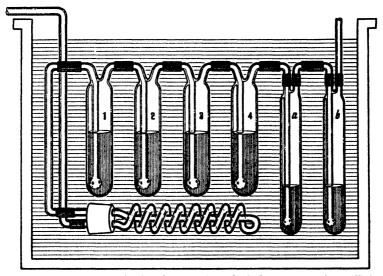


Fig. 1. Bubbler tube train for determining alcohol vapor tension. Tubes 1 to 4 contain the alcohol solution being studied; Tubes a and b, 50 per cent sulfuric acid for absorbing alcohol from the air which has passed through the four equilibrating tubes.

third alcohol absorption tube, but it caught no alcohol. After waiting 30 minutes to permit the bubbler tube contents to reach the bath temperature, air is passed through the tubes at the rate of about 100 cc. per minute. Atmospheric pressure in Tube 4 is maintained by using pressure at the inlet of the train and sufficiently reduced pressure in the air-collecting burette. The volume of air used is measured at room temperature and corrected to the temperature of the bath. 1 cc. of the dichromate solution is then added to Tubes a and b, the contents are mixed, and the tubes heated in boiling water for 10 minutes. After cooling, the excess dichromate is titrated in the usual way, being stirred by means of a stream of air through the inlet.

Method 2—The equilibrating apparatus is shown in Fig. 2 and a portion

of the heated exit tube in Fig. 3. The rotating cylinder (A) has a capacity of about 500 cc., and during the equilibration period it contains about 100 cc. of the alcohol solution. Bearings for cylinder A are provided by the standard taper joint at the left and a ball race at the right. A spring between the ball race and the body of the cylinder prevents leakage at the standard taper joint, which is lubricated with vaseline. Since alcohol is much less soluble in vaseline than in water, the small amount of lubricant used causes no appreciable loss of alcohol. The clamp on the tube leading to the fluid reservoir (D) is closed during the half hour equilibration period. At intervals during this period the exit tube (B) is raised above the water level and the cap removed to insure atmospheric pressure within the cylinder.

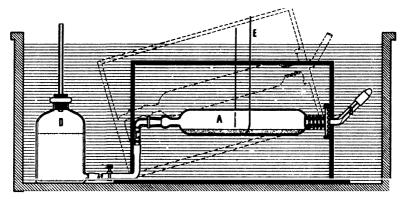


Fig. 2. Rotating cylinder for equilibrating air with blood, water, etc., containing alcohol. A, rotating cylinder; B, cylinder exit tube; C, L-tube for connecting cylinder with reservoir; D, reservoir for alcohol solution; E, belt.

With this apparatus it is more convenient to have the alcohol absorption portion outside the bath. As pointed out by Thomas (3) and Dobson (4), one must keep the temperature of the exit tube equal to, or higher than, that of the bath. Our exit tube (outside diameter, 0.5 mm.) is heated internally with a piece of 26 gage nichrome wire, as shown in Fig. 3, through which a current of about 0.9 ampere flows, heating the tube to about 45°. The protruding loops of the heating wire prevent moisture condensation at the junctions of this exit tube with the rotating cylinder and the alcohol absorption tube. Experiments with and without the current flowing showed that no alcohol was oxidized at the temperature of the wire. The alcohol absorption tube is of the type shown as Tube a in Fig. 1. It is cooled in ice water, and, as shown by tests, a single tube serves to catch all the alcohol. Prior to analyzing the absorption tube contents for alcohol, any moisture plus alcohol which may have condensed just beyond the

heated wire in the inlet is washed down with about 0.5 cc. of the 50 per cent sulfuric acid and then by drawing fluid up into the inlet once or twice. The remainder of the analysis is the same as in Method 1.

Method 3—This method is the result of our observation (1) that, if air is discharged through a gas-distributing disk and the tiny bubbles allowed to pass through a 2 inch layer of the alcohol solution, the air attains complete equilibrium in one passage. The apparatus used is shown in Fig. 4.

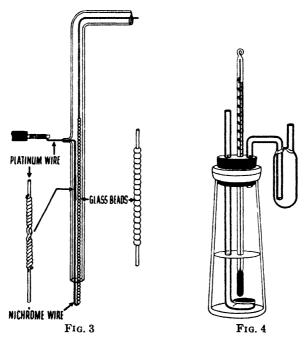


Fig. 3 Arrangement for internal heating of exit tube and its junction with other apparatus

Fig. 4. Gas-distributing disk apparatus for equilibrating air with water, urine, or blood containing alcohol.

The cylinder is an 8 ounce urine bottle. A 2 cm. gas-distributing disk (Jena, porosity 1, or Corning, porosity C) is used. The glass trap is connected to the alcohol absorption tube by means of the heated exit tube (Fig. 3). To avoid significant loss of alcohol when the liquid being tested is used for several runs, a second equilibrating apparatus, identical with the first except for the trap, is inserted before the one containing the test solution. The first apparatus contains a water solution of alcohol having a vapor tension of alcohol slightly below, or somewhat above, the test solution. This permits one to approach equilibrium from below, or from above.

In analyzing urine we add 2 or 3 drops of an antifoam fluid which one of us (E. G. B.) developed. It is liquid petrolatum containing 6 per cent of zinc stearate. Tests with water solutions of alcohol showed that the antifoam fluid causes no change in the vapor tension of alcohol. This antifoam material also permits the use of blood in this apparatus. When blood is used, the interior of the bottle is lightly coated with paraffin and the upper parts of the thermometer and gas-distributing tubes are coated with liquid petrolatum. In this way blood may be aerated almost as rapidly as water.

100 cc. of the liquid to be tested are placed in the aeration bottle with the trap outlet, and a like volume of the desired water solution of alcohol in the first bottle. They are connected together and the trap outlet is connected to the heated exit tube. Next the bottles are submerged in the constant temperature bath. When the thermometers in the bottles read the same as the bath thermometer the aeration is conducted, the air being forced through by means of an atomizer bulb. Before connecting the alcohol absorption tube the train and exit tube are flushed out with two or three bulbs full of air. The alcohol absorption tube is the same as that used in Method 2, and is cooled in a beaker of ice water. While an air pressure of about 20 mm. of mercury is required to force the air through each gasdistributing disk, this produces no pressure in the bottle containing the test solution, which is kept at atmospheric pressure by lowering the pressure in the gas burette just sufficiently to balance that required to run gas through the alcohol absorption tube. The remainder of this method is identical with Method 2.

Results

Alcohol Partition Ratio for Air-Water (A/W)—The temperatures employed were 1°, 37°, and intervals of 5° from 5–40°. The alcohol concentrations used ranged from 1 to 20 mg. per cc. of water. Method 1 was used for the major portion of the determinations, but a number of them were run by Methods 2 and 3. No differences could be observed in the results by the three methods. For a given temperature two or more concentrations of alcohol were used in order to see whether Henry's law is obeyed in the range of alcohol concentration used. The results indicated that it is obeyed. Thus, at 20° the amount of alcohol per cc. of water and the figure for $k_{A/W} \times 10^3$ were, respectively, 2 mg., 0.157; 5 mg., 0.154; and 10 mg., 0.154. At 30° the corresponding figures were 1 mg., 0.310; 2 mg., 0.311; 5 mg., 0.311; and 10 mg., 0.311. At 37° the figures were 1 mg., 0.472; 2 mg., 0.466; 5 mg., 0.475; and 10 mg., 0.466.

With some of the solutions equilibrium was approached both from below and also from above. The latter was accomplished in Method 1 by placing twice the concentration of alcohol in Tube 1 as in Tubes 2 to 4, with a similar arrangement in Method 3. If the mean of $k_{A/W} \times 10^8$ is represented by < where the equilibrium is approached from below and by > where equilibrium is approached from above, the results obtained for three temperatures were as follows: 25°, < = 0.217, > = 0.219; 37°, < = 0.471, > = 0.472; 40°, < = 0.567, > = 0.562.

The chief experimental details and the results of these determinations are summarized in Table I. In order to conserve space, the figures for individual determinations are not given. The fourth column gives the mean of all determinations for each temperature, and the standard and maximum deviations from this mean appear in the fifth and sixth columns.

erature No. of determinations		Concentration of alcohol in	Mean kA/W	Deviation from mean		
	water × 102*		Standard	Maximum		
·C.		mg. per cc.		per cent	per cent	
1	6	5-20	0.035	3.5	4.4	
5	12	10-20	0.046	4.3	7.1	
10	12	10-20	0.073	2.2	3.8	
15	12	5-10	0.107	1.6	3.1	
30	18	2-10	0.155	2.0	3.9	
25	13	2-10	0.217	2.4	3.8	
30	23	1- 5	0.310	1.7	3.2	
35	6	2	0.418	2.1	3.6	
37	28	1-10	0.470	2.2	3.2	
10	7	2	0.562	1.8	3.4	

Table I

Partition Ratio of Alcohol between Air and Water

Fig. 5 gives the curve for the values found by us for $k_{A/W} \times 10^3$ at temperatures of 1–40°. In Fig. 5 we have also plotted the results published by other investigators. Haggard and his colleagues have reported their results as the partition ratio water-air. We have converted them into the corresponding figures for air-water $\times 10^3$. For those publications reporting the partial pressure of alcohol we have translated these figures into mg. of alcohol per liter of air at the temperature used, and then from this and the corresponding water concentration into $k_{A/W} \times 10^3$.

Alcohol Partition Ratio for Air-Blood (A/B)—These determinations were run at seven temperatures from 10–40°. Human blood was used in all of the experiments except those run at 40° in which beef blood was used. A determination of hematocrit or the water content was carried out on all

^{*} k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/(weight of alcohol per unit volume of water). When the concentration of alcohol in the water is 1 mg. per cc., the value of $k_{A/W} \times 10^3$ equals mg. of alcohol per liter of air.

blood samples to make sure that they were of normal composition. Data from two blood samples with low solid content are not included in our averages. The anticoagulant used was sodium fluoride in a concentration of about 0.5 per cent in the blood. The alcohol added to the blood varied in strength from 10 per cent in water to absolute. The resulting dilution of

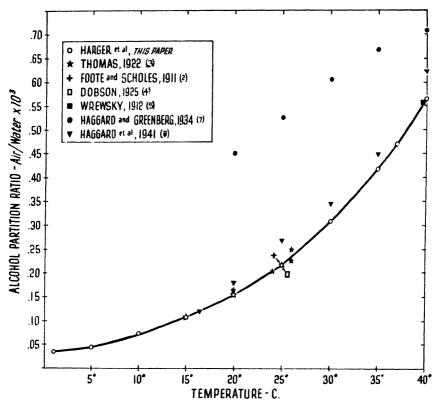


Fig. 5. Partition ratio of alcohol between air and water for temperatures of 1-40°. At 15°, 20°, and 25° our results and those of Thomas are almost identical. At 25° the results of Foote and Scholes and of Dobson are practically the same as that of Thomas and ours.

the blood was never greater than 2 per cent and was usually much less than this. After adding the alcohol and mixing well, the blood was allowed to stand at least a half hour before being used in order to insure penetration of the alcohol into the corpuscles.

Method 3 was used for most of the determinations, Method 2 being employed for some of them. The results with the two methods agreed well. Since blood is more viscous than water and might equilibrate with air less

readily than water, we approached equilibrium both from below, and from above, with all the blood samples. For each blood sample we ran about the same number of tests from above as from below.

The details and results of our air-blood studies are summarized in Table II. As shown in the next to last column, whether equilibrium was approached from below or from above made very little difference in $k_{A/B}$. The last column in Table II gives the ratio of $k_{A/B}$: $k_{A/W}$. This ratio was found to be approximately 1.25:1.00 for all temperatures employed. This is not far from the reciprocal of the ratio of water content per unit volume of the two fluids.

Temperature det	No of determina-	Concentra- tion of alcohol in	Mean k _{A/B} × 10 ²⁺	Deviation from mean		Change < to >†	kA/B:kA/W
	tions	blood			Maxi- mum		
•		mg. per cc		per cent	per cent	per cent	
10	5	8 69	0.092	1.3	2.0	+0.1	1.245
20	7	8.0	0.194	2.6	3.5	+3.0	1.251
30	7	2 0	0.393	2.0	3.1	-0.5	1.263
35	9	2 0	0.515	1.4	2.2	0.0	1.245
37	19	1.0 - 2.0	0.591	1.7	3.1	+1.5	1.255
40	7	2 0	0.703	1.0	1.6	-1.1	1.250

TABLE II
Partition Ratio of Alcohol between Air and Blood

A sample of sheep blood having a hematocrit¹ of 38 and a plasma protein content of 5.8 per cent, when run at 37°, was found to have a value of $k_{A/B}$ × 10³ of 0.547 for the whole blood and 0.509 for the plasma. These results are not included in Table II.

The curve showing our values for $k_{A/B} \times 10^3$ at the various temperatures used is given in Fig. 6. In this chart we have also plotted the figures for $k_{A/B} \times 10^3$ appearing in the literature.

Alcohol Partition Ratio for Air-Urine (A/U)—Equilibration Method 3 was used for urine. Determinations were made at five temperatures from 10-37°. Table III summarizes the data for these experiments. As shown in the last column, the ratio for $k_{A/U}:k_{A/W}$ varied from 1.08:1.00 to 1.09:1.00.

^{*} k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/(weight of alcohol per unit volume of blood). When the concentration of alcohol in the blood is 1 mg. per cc., the value of $k_{A/B} \times 10^3$ equals mg. of alcohol per liter of air.

 $[\]dagger < = k_{A/B}$ with equilibrium approached from below. $> = k_{A/B}$ with equilibrium approached from above.

¹ Per cent of total blood volume occupied by cells.

A sample of urine from a case of diabetes insipidus, having a specific gravity of 1.005, gave 0.474 as $k_{A/U} \times 10^3$ at 37°. This is close to the value of $k_{A/W} \times 10^3$ at 37°, which we found to be 0.470. The results for this urine are not included in Table III.

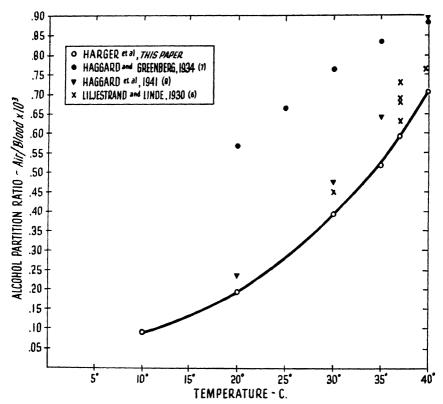


Fig. 6. Partition ratio of alcohol between air and blood for temperatures of 10-40°.

Aerometric Method for Determining Alcohol in Water, Urine, Etc.

Apparatus and Reagents—The fluid to be analyzed is brought to room temperature and equilibrated with air in the apparatus shown in Fig. 4 and the air leaving the trap is analyzed for its alcohol content. The air analysis may be done by the dichromate method mentioned above. However, it was found that the permanganate method of Harger, Lamb, and Hulpieu (18) gives satisfactory results, and, since it uses a smaller weight of alcohol and requires only about 2 minutes to run, it was chosen for this aeration procedure. Only reaction Tube B which is shown in Fig. 4 of this 1938 paper (18) is used.

Following the publication of this permanganate method (18) we have added a color-matching tube, placed next to the reaction tube, in order to read the end-point of the reaction more accurately. The matching fluid is made by mixing 27 cc. of 5 per cent cobalt sulfate solution,² 1 cc. of 1 per cent potassium dichromate, and 89 cc. of water. Since this matching fluid contains a trace of pink color, which adds to the precision of reading, a very small amount of permanganate remains at the end-point. This results in a slightly lower alcohol equivalent for 1 cc. of 0.05 n KMnO₄, which is 0.169 mg. of alcohol instead of 0.175 as given in our original paper. The figure for the alcohol equivalent of the permanganate was determined by titration with a solution of alcohol in the same concentration of sulfuric acid

Deviation from Concentramean No. of Change < to >† tion of Mean k_A/U × 1030 kA/U: kA/W Temperature determinaalcohol in tions urine Stand-Maxiard mum •c. mg. per cc. per cent per cent per cent 2.1 2.5 1.09 10 5 8.0 0.080-1.620 8 4.0 0.1691.4 2.9 1.09 2.1 2.0 - 4.00.33830 14 1.6 0.0 1.09 37 21 1.64 - 2.00.5092.3 3.4 1.08

TABLE III

Partition Ratio of Alcohol between Air and Urine

containing 0.2 mg. of alcohol per cc. The illumination of the reaction tube and matching tube should be from the rear and not from the front or side.

No constant temperature bath is needed in this method of analysis because it is conducted at room temperature and this will not change appreciably during the period of about 2 minutes required for running the analysis. To facilitate cleaning and drying the trap and its connections between runs, a trap closed with a 2-hole rubber stopper is substituted for the all-glass one shown in Fig. 4.

Procedure

100 cc. of the alcohol solution are placed in the equilibrating bottle (Fig. 4). With urine, 2 or 3 drops of the antifoam liquid are added. The

^{*} k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/ (weight of alcohol per unit volume of urine). When the concentration of alcohol in water is 1 mg. per cc., the value of $k_{A/U} \times 10^3$ equals mg. of alcohol per liter of air.

 $[\]dagger < = k_{A/U}$ with equilibrium approached from below. $> = k_{A/U}$ with equilibrium approached from above.

² 5 gm. of CoSO₄·7H₂O are dissolved in 100 cc. of water and then 0.5 cc. of 50 per cent sulfuric acid added.

fluid is brought to room temperature by cooling, or warming, the bottle with water. After flushing out the bottle and trap with one or two bulbs full of air the trap outlet is connected to the reaction tube and this in turn to the gas burette. With a slightly reduced pressure in the gas burette, air is forced through the apparatus at the rate of 200 to 500 cc. per minute until about four-fifths of the permanganate color has disappeared. The remainder of the test is conducted slowly, running the air through intermittently in spurts of 10 to 20 cc. and waiting about 5 seconds between spurts of air. The air passage is stopped at the point at which the color in the reaction tube reaches that of the matching fluid or is just perceptibly lighter. The temperature of the fluid being tested is read with an accuracy of 0.1°. No correction in gas volume is needed, since the temperatures are identical in the equilibrating bottle and the gas burette.

For greater accuracy one may conduct one or two more determinations with the same alcohol solution. The contents of the reaction tube are drained and new reagents added without rinsing the tube with distilled water unless the end-point is distinctly passed in the previous test.

When solutions in the equilibration bottle are changed, the stopper and upper portion of the glass parts should be wiped dry. Moisture here will change the alcohol content of the air which has passed through the fluid. Also, the trap and its glass connections and the rubber tube connecting it to the reaction tube must remain dry. Although the lower portion of the bubbler inlet in the reaction tube is wet with acid, this will cause no error if the level of acid in this tube reaches the same point between intermittent passages of air through the apparatus.

Tests showed that so long as the last part of the passage of air is conducted slowly the time for completing the analysis is not important. Determinations conducted when a period of 5 minutes elapsed between the consumption of most of the permanganate and the completion of the test yielded the same results as tests completed in 2 minutes from the start.

Calculation

Water Solutions of Alcohol—The weight of alcohol required to decolorize the permanganate is constant, 0.169 mg., the variable in this analysis being the volume of the equilibrated air containing this weight of alcohol. This is the volume $V_{\rm obs}$ read in the gas burette. It is inversely proportional to the concentration of alcohol in the air, and also in the water. Next, one calculates the volume of air (V_1) which would have been required at this temperature if the water contained 1 mg. of alcohol per cc. The formula for this calculation is

$$V_1 = \frac{0.169 \times 1000}{k_{A/W} \times 10^3}$$

the result being in cc. The value of $k_{A/W} \times 10^{3}$ is obtained from the fourth column of Table I or the curve of Fig. 5. Then, V_{1}/V_{cbs} . = mg. of alcohol per cc. of the aqueous solution of alcohol analyzed. A table giving values of V_{1} for 0.1° intervals from about 15–35° is convenient if one has many analyses to make.

Urine—The calculation is the same as for water, except that the value $k_{A/U}$ replaces $k_{A/W}$. An alternative method is to use the calculation for water and divide the result by 1.09, which is the average ratio of $k_{A/U}$: $k_{A/W}$.

Results Obtained with Method

Twenty water solutions of alcohol, with concentrations ranging from 0.91 to 4.72 mg. per cc., were submitted to an assistant who analyzed them by this method. His average error was ± 2.2 per cent, with maximum errors of +5.1 and -5.8 per cent. He also analyzed sixteen urine samples containing 0.49 to 3.35 mg. of alcohol per cc., running duplicate analyses with each sample. The average error for the thirty-two determinations was ± 4.0 per cent, with maximum errors of +7.1 and -9.0 per cent. The composition of all samples was unknown to the analyst.

Use of Aerometric-Permanganate Analysis Plus Dichromate Reduction As Identification Test for Ethyl Alcohol

The fluid or tissue being analyzed for alcohol is distilled as usual. A portion of the distillate is analyzed for alcohol by the dichromate method used in the first part of this study. Assuming that the only reducing substance present in the distillate is ethyl alcohol, its concentration in the distillate is calculated and the result expressed as mg. of ethyl alcohol per cc., which we will designate as $C_{\mathbf{K_1Cr_2O_7}}$. A second portion of the distillate is run by the aerometric-permanganate method described in the preceding section, with a period of about 3 minutes to reach the end-point. This result is also calculated as the concentration of ethyl alcohol expressed as mg. per cc., which we will call $C_{\mathtt{aer.-KMnO_4}}$. If the reducing material present in the distillate is all ethyl alcohol, $C_{\mathtt{K_1Cr_2O_7}}$ will equal $C_{\mathtt{aer.-KMnO_4}}$. If the two are unequal, a part, or all, of the reducing substance is not ethyl alcohol.

This double analysis procedure was applied to a number of aliphatic alcohols and to ether and acetone. The results obtained for the $C_{\rm K_2Cr_2O_7}$: $C_{\rm aer..KMnO_4}$ ratio are given in Table IV. Table IV also includes our results for the dichromate equivalent of the different compounds. With all of the alcohols tested, except ethyl alcohol, the result obtained with the aerometric-permanganate analysis varied somewhat with the time required to reach the end-point, smaller volumes of air (i.e. less alcohol) being required if the time interval of analysis was 5 to 10 minutes instead of the usual time of 3 minutes. Methyl alcohol in water has about the

same $k_{A/W}$ as ethyl alcohol. The high $C_{\text{K}_2\text{Cr}_2\text{O}_7}$: $C_{\text{aer}.\text{KMnO}_4}$ ratio obtained with methyl alcohol is due to differences in its oxidation with dichromate and with permanganate. In the dichromate reaction ethyl alcohol is oxidized to acetic acid, while methyl alcohol goes to carbon dioxide and water (10). In the reaction with permanganate in the presence of 16 N sulfuric acid our evidence indicates that ethyl alcohol still goes to acetic acid, while methyl alcohol is probably oxidized to formaldehyde. This would mean that in the dichromate reaction methyl alcohol consumes 3 times as much

Table IV

Comparison of Results from Dichromate Analysis with Those from Aerometric-Permanganate Analysis for Certain Aliphatic Alcohols, Ether, and Acetone

Alcohol	Alcohol equivalent of 1 cc of 0 0434 N k ₂ Cr ₇ O ₇	Ratio, $\frac{C_{\text{K}_2\text{Cr}_2\text{O}_7}}{C_{\text{aer}} - \text{KMnO}_4}$		
	mg			
Ethyl	0 500	1 00		
Methyl	0 230	2 55		
Propyl	0 493	0 90		
Isopropyl	1 047	0 63		
n-Butyl	0 762	0 59		
Isobutyl	0 584	0 73		
n-Amyl	0 746	0 32		
Isoamyl	0 678	0 62		
Tertiary amyl	0 722	0 55		
Ether	0 403	0 013		
Acetone	16 6	No reduction of perman- ganate reagent		

^{*} C_{K2Cr2O7} = dichromate result calculated as ethyl alcohol $C_{\text{aer.-KMnO_4}}$ = aerometric-permanganate result calculated as ethyl alcohol

oxygen as in the permanganate reaction. We have found that both ethyl alcohol and acetaldehyde rapidly reduce the permanganate reagent, while acetic acid causes no reduction whatever. In a large scale experiment it was shown that no carbon dioxide was produced. On account of the great excess of sulfuric acid and the presence of intermediate oxides of manganese (18) we did not attempt to identify acetic acid in the solution resulting from the reaction.

With the other alcohols studied the variation of the $C_{\text{K}_2\text{Cr}_2\text{O}_7}$: $C_{\text{ner..KMnO}_4}$ from that of ethyl alcohol is largely due to the fact that these alcohols have a higher $k_{A/W}$ than that of ethyl alcohol. This is also particularly true with ether, the $k_{A/W}$ of which is more than 100 times that of ethyl alcohol.

DISCUSSION

Larger scale operations for our determinations of the air-liquid alcohol partition ratio would probably have yielded slightly more precise results. However, we believe that any errors in the results obtained are not greater than 1 or 2 per cent.

While the alcohol partition ratio for air-water is constant for a given temperature, the ratios for air-blood and air-urine are subject to some fluctuations, which are approximately inversely proportional to the water content of these fluids (19). This is demonstrated by our results with sheep blood having somewhat low hematocrit and plasma protein values and also with diabetes insipidus urine. Air equilibrated with blood is really a three phase system, air, plasma, and cells, the three having the same alcohol vapor tension. Kunkele (20) obtained serum-clot alcohol ratios varying from 1.07:1.00 to 1.15:1.00, averaging 1.11:1.00 (weight-volume). A few analyses by us gave plasma-whole blood alcohol ratios ranging from 0.93:1.00 to 1.17:1.00 and averaging 1.05:1.00. These results would indicate that bloods with an abnormal cell volume might vary from normal bloods in their $k_{A/B}$ values as much as 5 to 10 per cent. Since variations in the alcohol vapor tension of bloods having the same concentration of alcohol would probably change the alcohol concentrations of breath and brain equally, it would appear that, for individuals with an abnormal water content of blood, breath analysis might predict the level of brain alcohol better than would analysis of blood.

Further evidence that the revised air-liquid alcohol partition ratio figures reported by Haggard et al. in 1941 (8) are still incorrect, at least in part, is shown by the variations they found in the relative values for $k_{A/B}$: $k_{A/W}$. According to their data these vary from 1.29:1.00 at 25° to 1.44:1.00 at 35° and 40°. The factors which cause the vapor tension of alcohol in blood to be higher than that in water should not change 12 per cent in a temperature interval of 10°. Also, we can see no theoretical reason why the alcohol vapor tension of normal blood should ever be 44 per cent greater than that of water having the same concentration of alcohol.

The $k_{A/B}$ ratios reported by Liljestrand and Linde (6) average about midway between ours and those reported by Haggard *et al.* in 1941 (8). If Liljestrand and Linde used normal bloods, the variation in their four figures for 37° indicates some rather larger errors.

For predicting the blood alcohol level from breath analyses the $k_{A/B}$ at 34° is important. As pointed out by Winslow, Herrington, and Nelbach (21), studies by various investigators agree that the average temperature of expired air is 34°. Liljestrand and Linde (6) stated that the temperature at which alveolar air leaves the mouth will control the partition ratio of

alcohol between breath and body fluid. Our determinations showed that at $34^{\circ} k_{A/B} \times 10^{3}$ is 0.493, which gives a blood-air ratio close to 2000:1. Simultaneous analyses of human breath and blood reported by Liljestrand and Linde (6), Harger, Lamb, and Hulpieu (18), Jetter, Moore, and Forrester (15, 22), and Fabre and Leheuzey (23) all point to a blood-alveolar air ratio which approximates 2000:1 and is not in agreement with the ratios given by Haggard and colleagues which were 1150:1 in 1934 (7) and 1300:1 in 1941 (8). This matter will be more fully discussed in a later paper.

In the use of our aerometric-permanganate method, smaller volumes of the alcohol solution may be employed by decreasing the diameter of the fluid container. With a cylinder having a diameter of about 4 cm., a 30 cc. sample is sufficient, and by employing an Allihn tube with fritted disk³ as little as 10 cc. of the alcohol solution may be used. However, when analyzing 10 cc. samples one should correct the result obtained for the drop in dissolved alcohol during the aeration.

SUMMARY

- 1. We report results of determinations of the partition ratio of alcohol for air-water (A/W) at 1–40°, air-blood (A/B) at 10–40°, and air-urine (A/U) at 10–37°. Three methods of equilibration were used and equilibrium was approached both from above and from below. The range of alcohol concentration used was from 1 to 20 mg. per cc. of liquid, in which range Henry's law was found to be obeyed.
- 2. At each of the temperatures used the relative values of the air-liquid partition ratios, with $k_{A/W}$ expressed as unity, were 1.25 for $k_{A/B}$ and 1.09 for $k_{A/U}$.
- 3. Our results for $k_{A/W}$ for alcohol are in substantial agreement with those of Foote and Scholes, Wrewsky, Thomas, and Dobson, but they are definitely lower than most of the 1941 revised figures of Haggard *et al.*
- 4. Our results for $k_{A/B}$ for alcohol are much lower than the 1941 figures of Haggard *et al.* and somewhat lower than those of Liljestrand and Linde.
- 5. At 34°, the temperature at which alveolar air is exhaled, we found that $k_{A/B} \times 10^8$ is 0.493, which would indicate a ratio of about 2000:1 for alcohol concentrations in blood to expired alveolar air.
- 6. A method is described for the rapid analysis of air equilibrated with a solution of alcohol, from which one may employ the partition ratio at that temperature to calculate the concentration of alcohol in the solution.
- 7. It was shown that a comparison of the result from dichromate oxidation with that obtained by an aerometric-permanganate analysis will serve to differentiate ethyl alcohol from many other reducing substances.

⁸ Corning No. 37730-30M with lower tube bent into a U.

BIBLIOGRAPHY

- Harger, R. N., Bridwell, E. G., and Raney, B. B., Proc. Am. Soc. Biol. Chem., J. Biol. Chem., 128, p. xxxviii (1939).
- 2. Foote, H. W., and Scholes, S. R., J. Am. Chem. Soc., 33, 1309 (1911).
- 3. Thomas, R., J. Soc. Chem. Ind., 41, 34 (1922).
- 4. Dobson, J. E., J. Chem. Soc., 127, 2866 (1925).
- 5. Wrewsky, M., Z. physik. Chem., 81, 1 (1912).
- 6. Liljestrand, G., and Linde, P., Skand. Arch. Physiol., 60, 273 (1930).
- Haggard, H. W., and Greenberg, L. A., J. Pharmacol. and Exp. Therap., 52, 150 (1934).
- Haggard, H. W., Greenberg, L. A., Miller, D. P., and Carroll, R. P., J. Lab. and Clin. Med., 26, 1527 (1941).
- Landolt-Börnstein physikalisch-chemische Tabellen, Berlin, 5th edition, 1, 762 (1923).
- 10. Harger, R. N., J. Lab. and Clin. Med., 20, 746 (1935).
- 11. Jetter, W. W., Quart. J. Studies Alc., 2, 512 (1941).
- 12. Methods for laboratory technicians, U. S. War Department Technical Manual TM8-227, 255 (1946).
- 13. Cavett, J. W., J. Lab. and Clin. Med., 23, 543 (1938).
- 14. Levine, H., and Bodansky, M., Am. J. Clin. Path., Tech. Suppl., 4, 103 (1940).
- Jetter, W. W., Moore, M., and Forrester, G. C., Am. J. Clin. Path., Tech. Suppl.,
 75 (1941).
- 16. Fisk, V. B., and Nelson, V. E., Proc. Iowa Acad. Sc., 48, 207 (1941).
- 17. Greenberg, L. A., Quart. J. Studies Alc., 6, 135 (1945).
- Harger, R. N., Lamb, E. B., and Hulpieu, H. R., J. Am. Med. Assn., 110, 779 (1938).
- 19. Harger, R. N., Hulpieu, H. R., and Lamb, E. B., J. Biol. Chem., 120, 689 (1937).
- 20. Kunkele, F., Deut. Z. ges. gerichtl. Med., 28, 241 (1936).
- Winslow, C. E. A., Herrington, L. P., and Nelbach, J. H., Am. J. Hyg., 35, 27 (1942).
- 22. Jetter, W. W., and Forrester, G. C., Arch. Path., 32, 828 (1941).
- 23. Fabre, R., and Leheuzey, P., Bull. Soc. chim. biol., 26, 49 (1944).

STUDIES ON THE METABOLISM OF THIOUREA

I. DISTRIBUTION AND EXCRETION IN THE RAT OF THIOUREA LABELED WITH RADIOACTIVE SULFUR*

By JOHN SCHULMAN, JR.,† AND RICHARD P. KEATING;

(From the Los Alamos Scientific Laboratory of the University of California, Los Alamos, New Mexico)

(Received for publication, September 26, 1949)

The goitrogenic action of thiourea and its derivatives was first described in 1943 (1, 2). It is known that this series of drugs, while not affecting the uptake of iodide ion by the thyroid gland, interferes with the formation of organic iodine (3-5). The mechanism of this action is unknown. Williams, using a chemical method of analysis, found a significant concentration of thiouracils in the thyroid and pituitary glands (6, 7). A new method of study of this problem was afforded by the preparation of thiourae labeled with S³⁵.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were fed a diet of Purina laboratory chow containing 5 parts per million of iodine. 4 to 12 week-old animals were used to minimize the toxicity of thiourea (8, 9). The thiourea used in the earlier experiments had a specific activity of 12,000 to 28,000 counts per second per mg. in the counters used. In small organs with little evidence of concentration, the counts obtained with this material were so low (0.1 to 0.3 counts per second) that the results were in doubt. Therefore, when material of much higher specific activity (1,500,000 counts per second per mg.) was obtained, some of the experiments were repeated with this material.

Each animal received 1 mg. of thiourea in 1 ml. of saline intraperitoneally and was placed in a metabolism cage. Urine and feces were collected. Expired air was passed through three traps containing 0.1 n hydrochloric acid, 0.1 n sodium hydroxide, and 3 per cent hydrogen peroxide successively. The rats were killed with ether at intervals of 6, 24, 48, and 72 hours following injection. Samples included the intact thyroid

^{*}This document is based on work performed under contract No. 7405-eng-36 for the Atomic Energy Commission. Read in part before the Association for the Study of Internal Secretions at Atlantic City, June 4, 1949.

[†] Captain, Medical Corps, Army of the United States.

[‡] Present address, Department of Radiobiology, Army Medical Department Research and Graduate School, Washington, D. C.

and pituitary glands, portions of other tissues ranging in wet weight from 0.2 to 0.6 gm., and aliquots of urine and dried feces. After tissue samples were collected, the remainder of the body was dissolved in an ashing mixture and an aliquot was taken for ashing. 14 mg. of sodium sulfate were added to each sample as a carrier before ashing. Ashing was carried out by the method of Boursnell et al. (10). The ash was dissolved in dilute hydrochloric acid, transferred to a 40 ml. centrifuge cone, precipitated with 5 ml. of 5 per cent barium chloride solution, and heated in an oven at 90° for 1 hour. The precipitate was centrifuged, washed twice with water and once with 95 per cent ethyl alcohol, and plated on a copper disk ac-

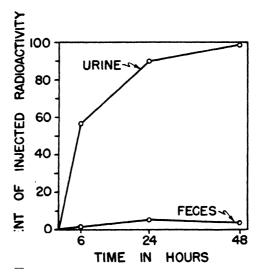


Fig. 1. Excretion of radioactivity in urine and feces following injection of labeled thioures.

cording to the method of Hendricks et al. (11). The samples were counted with a mica window Geiger-Müller counter. A correction for thickness was applied by use of an experimentally determined absorption curve.

To determine the distribution of the sulfide ion when injected intraperitoneally, a group of six rats was injected with an aqueous solution of barium sulfide labeled with S³⁵ containing an undetermined quantity of carrier. Similarly, the distribution of the sulfate ion was determined by injecting rats with a saline solution of carrier-free sulfuric acid labeled with S³⁵. The rats were killed 24 hours after injection and the radioactivity of the thyroid glands was determined as above.

Urine inorganic sulfate and ethereal sulfate were separated chemically according to Folin's method (12). The radioactive urinary components

were also studied by one-dimensional filter paper partition chromatography. 0.05 ml. samples of urine were placed on 60×2 cm. strips of Whatman No. 1 filter paper and chromatograms were developed in the following systems: (1) butanol and water; (2) butanol and 1 n hydrochloric acid; and (3) butanol and 3 per cent ammonium hydroxide. Control chromatograms were developed in the same systems, with radioactive thiourea in water and in experimental urine. After development, radioautographs were made on 14×17 inch Blue Brand x-ray film.

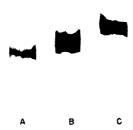


Fig. 2. Radioautograph of one-dimensional filter paper chromatogram of urine from a rat injected with radioactive thiourea. Test solutions were placed on each filter paper strip at the dotted line. Strip A contained experimental urine, B contained experimental urine to which radioactive thiourea was added, and C contained radioactive thiourea in water.

Results

The excretion of radioactivity after the injection of S³⁵-labeled thiourea is shown in Fig. 1. Over 98 per cent of the activity was excreted in the urine within 48 hours. Fecal excretion was slight and may have been due in part to contamination with urine. No radioactivity was present in the expired air.

Chromatograms of the urine showed a single major band, the migration of which was similar to that of thiourea. This indicates that the major portion of thiourea was excreted unchanged. Fig. 2 shows the radioautograph of a typical chromatogram. Chemical analysis showed that 6.2 per cent of the radioactivity in the urine was present as inorganic sulfate and 5.9 per cent as ethereal sulfate.

Table I shows the distribution of radioactivity after the administration of labeled thiourea. Each value represents the average of determinations

Table I

Concentration of Radioactivity in Rats Injected with Radioactive Thiourea

m.	Tissue concentration, per cent dose per gm.					
Tissue	6 hrs	24 hrs.	48 hrs	72 hrs.		
Carcass	$0.288^* \pm 0.04$	$45.0.0707 \pm 0.013$	$3.0.0276 \ddagger \pm 0.0020$	60.0285 § ± 0.0117		
Thyroid	$2.99* \pm 0.35$	$5.2.20\dagger \pm 0.41$	$1.44^{+}\pm 0.14$	$ 1.278 \pm 0.233$		
Kidney	-0.396 ± 0.13	380.140 ± 0.020	0.113 ± 0.033			
${f A}$ drenal	0.241 ± 0.07	70.989 ± 0.010	0.0557 ± 0.0266			
Bone	0.184 ± 0.05	60.0549 ± 0.0079	0.0404 ± 0.0039	1		
Brain	0.140 ± 0.03	$320.0111^{4} \pm 0.0028$	80.00762 ± 0.0055	1		
Colon	$^{-1}0.253 \pm 0.00$	$65.0.0777 \pm 0.0197$	$7.0.0430 \pm 0.0123$			
Heart	0.187 ± 0.04	50.0240 ± 0.0040	0.00937 ± 0.00179	0,		
Liver	0.242 ± 0.01	90.095 ± 0.020	0.0780 ± 0.0165			
Lung	0.256 ± 0.06	$5.0.0657 \pm 0.0144$	$\pm 0.0350 \pm 0.0087$			
Muscle	0.174 ± 0.04	70.0120 ± 0.0020	$5.0.00910 \pm 0.00643$	5		
Pituitary		0.0612 ± 0.0113	30.0128 ± 0.0035	1		
Skin	0.230 ± 0.12	80.0410 ± 0.0071	0.0545 ± 0.0048			
Small	1					
bowel	0.222 ± 0.05	$6.0.0494 \pm 0.0057$	0.0355 ± 0.0142	1		
Spleen	0.213 ± 0.07	40.0411 ± 0.0067	0.0337 ± 0.0013			
Stomach	0.158 ± 0.03	70.0483 ± 0.0174	$\pm 0.0312 \pm 0.0055$			
Testis	0.201 ± 0.08	$0.0.0174 \pm 0.0034$	$\pm 0.0107 \pm 0.0006$			

- * Seven animals used.
- † Eleven animals used.
- † Twelve animals used.
- § Nine animals used.
- || Eight animals used.
- ¶ Two animals used.

on three animals unless otherwise noted. The results are expressed as the per cent dose per gm. of tissue. The carcass value represents the average concentration in the body. The thyroid and kidney values are significantly greater than that of the carcass. The maximum value for the thyroid gland was reached at 6 hours.

Fig. 3 shows the data from the thyroid gland expressed as the ratio of the concentration of radioactivity in the gland to that of the average concentration in the body. This indicates the degree to which the sulfur injected as thiourea was concentrated in the thyroid gland. The standard error for each point is indicated on Fig. 3. These values were calculated by determining the ratios for each animal and then averaging them, not

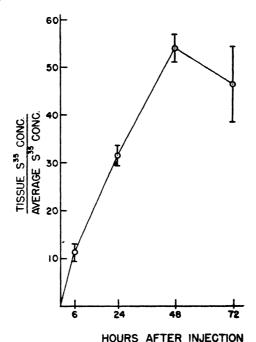


Fig. 3. Concentration of radioactivity in thyroid gland compared to average concentration of radioactivity in the body following injection of labeled thioures.

Table II

Concentration of Radioactivity in Rats Injected with Radioactive Barium Sulfide and

Sulfuric Acid

Injected material	Tissue	Tissue concentration at 24 hrs., per cent dose per gm.
Barium sulfide	Thyroid	
Sulfuric acid	Thyroid Carcass	0.213 ± 0.057

by comparing the average thyroid value and the average carcass value. This resulted in a smaller error. It will be noted that, although the maximum concentration in the thyroid was at 6 hours, the maximum relative concentration was at 48 hours.

Table II shows the distribution of radiosulfur when it was injected as BaS and H₂SO₄. In neither case was the concentration higher in the thyroid gland than the average in the body.

DISCUSSION

After the intraperitoneal injection of thiourea labeled with radiosulfur, significant concentrations of radioactivity were found in the kidney and thyroid gland. The concentration in the kidney can be explained by the finding that the kidney was the principal route of excretion of the drug.

The concentration in the thyroid gland is best seen when compared with the average concentration in the body. This relative concentration reached a maximum 48 hours after injection. This was later than the peak of the absolute concentration, which was reached 6 hours after injection, indicating that the sulfur was held in the thyroid gland at a time when the concentration in the body as a whole was falling.

The chemical nature of the sulfur which was concentrated in the thyroid gland cannot be determined from these data. Whether it was thiourea or some metabolic product of thiourea is not known. Possible metabolic products are the sulfide or sulfate ions. Neither of these ions was concentrated when injected as such.

It has been shown that the injected thiourea was rapidly excreted in the urine, most of it unchanged. However, the excretion of about 10 per cent of the injected sulfur as sulfate indicates partial metabolism of the compound.

The data in this paper differ slightly from those given in a previous report (13). With material of specific activity sufficiently high to give significant counting rates even in very small organs, it was found that the concentration of activity in the pituitary gland was no higher than the average in the body.

The mechanism of the goitrogenic action of thiourea is still unknown, although several hypotheses have been advanced (14-17). A valid hypothesis must take into account the concentration of sulfur in the gland following the injection of thiourea. The partial metabolism of the compound in the body may also be of importance in explaining this action.

SUMMARY

Thiourea labeled with radioactive sulfur was injected into rats.

98 per cent of the activity was excreted in the urine within 48 hours.

Of the radioactivity in the urine, 6 per cent was in the form of inorganic sulfate and 6 per cent was in the form of ethereal sulfate. The major portion of the thiourea was excreted unchanged.

There was a 55-fold concentration of radioactivity in the thyroid gland.

We are indebted to Miss Frances Sharp for technical assistance, to Dr. D. L. Tabern of the Abbott Laboratories, and Dr. A. R. Ronzio and C. Wayne Bills of this laboratory for the preparation of the thiourea used in these experiments.

BIBLIOGRAPHY

- Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., Endocrinology, 32, 210 (1943).
- 2. MacKenzie, C. G., and MacKenzie, J. B., Endocrinology, 32, 185 (1943).
- 3. Franklin, A. L., Chaikoff, I. L., and Lerner, S. R., J. Biol. Chem., 153, 151 (1944).
- 4. Taurog, A., Chaikoff, I. L., and Feller, D. D., J. Biol. Chem., 171, 189 (1947).
- 5. McGinty, D. A., and Sharp, E. A., Endocrinology, 39, 74 (1946).
- 6. Williams, R. H., Kay, G. A., and Jandorf, B. J., J. Clin. Invest., 23, 613 (1944).
- 7. Williams, R. H., and Kay, G. A., Arch. Int. Med., 80, 37 (1947).
- MacKenzie, J. B., and MacKenzie, C. G., Proc. Soc. Exp. Biol. and Med., 54, 34 (1943).
- 9. Dicke, S. H., and Richter, C. P., J. Pharmacol. and Exp. Therap., 83, 195 (1945).
- 10. Boursnell, J. C., Francis, G. E., and Wormall, A., Biochem. J., 40, 743 (1946).
- Hendricks, R. H., Bryner, L. C., Thomas, M. D., and Ivie, J. U., J. Phys. Chem., 47, 469 (1943).
- Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 78 (1937).
- 13. Schulman, J., Jr., and Keating, R. P., J. Clin. Endocrinol., 9, 664 (1949).
- Miller, W. H., Roblin, R. O., Jr., and Astwood, E. B., J. Am. Chem. Soc., 67, 2201 (1945).
- 15. De Robertis, E., and Grasso, R., *Endocrinology*, **38**, 139 (1946).
- 16. Astwood, E. B., Ann. New York Acad. Sc., 50, 419 (1949).
- Rawson, R. W., Albert, A., McArthur, J. W., Merrill, P., Lennon, B., and Riddell, C., Endocrinology, 39, 74 (1946).

STUDIES ON HYDROCARBONS STRUCTURALLY RELATED TO PHTHIOCEROL

SYNTHESIS OF THE LEVOROTATORY ENANTIOMORPH OF 4-METHYL-TRITRIACONTANE

By STINA STÄLLBERG-STENHAGEN AND EINAR STENHAGEN (From the Institute of Medical Chemistry, University of Upsala, Upsala, Sweden)

(Received for publication, October 14, 1949)

It has been shown previously (1, 2) that phthiocerane, the hydrocarbon derived from the wax alcohol phthiocerol present in the human and bovine types of tubercle bacilli (3-7), is a 4-methyl-substituted long chain hydrocarbon. The properties of phthiocerane were closely similar to those of synthetic DL-4-methyltritriacontane (1), but the former melted 0.5° above the melting point of the synthetic hydrocarbon, and there were slight differences in the appearance of the x-ray diffraction patterns. suggested that these differences might be caused by the presence of a small amount of a higher homologue (4-methylpentatriacontane) in the hydrocarbon derived from the natural product. Another suggested possibility was that phthiocerane, in spite of the absence of a detectable optical rotation (3), might be one of the enantiomorphs of 4-methyltritriacontane. seems likely that only one of the two possible configurations of the asymmetric carbon atom carrying the methyl group is present in the natural product. If the reduction of phthiocerol gives a racemic hydrocarbon, this would suggest that one of the functional groups (hydroxyl or methoxyl) of the alcohol is attached at, or situated in the α position to, the carbon atom carrying the methyl side chain. It was thus of interest to compare the properties of phthiocerane with those of one of the enantiomorphs of 4methyltritriacontane, and the synthesis of the levorotatory form has been undertaken, with p(-)-21-methyltetracosanoic acid (I), m.p. 60.6-60.8°. $[M]_{p}^{21} = -3.2^{\circ}$, as initial material. The synthesis of I is described in another communication (8). The optically active acid (I) was converted into D(-)-21-methyltetracosanol-1 (II) by reduction with lithium aluminum hydride in ether solution according to the method of Nystrom and Brown The further steps in the synthesis are analogous to those used in the synthesis of the racemic hydrocarbons (1), as shown in the accompanying flow sheet.

The molecular rotations of the alcohol (II), the corresponding iodide (III), D(-)-8-keto-30-methyltritriacontane (V), and the levorotatory enantiomorph of 4-methyltritriacontane (VI) are all of the order of -3° to -4° ; i.e., of the same order of magnitude as that of I. The observed

rotations are very small, however, and the molecular rotations cannot be given with great accuracy. In particular, the rotation for a 1 dm. tube of a 4.8 per cent solution of (-)-4-methyltritriacontane in chloroform is $\alpha_p^{22} = -0.03^\circ$, showing that if phthiocerane is optically active its activity might have easily escaped detection.

(V) D(-)-8-Keto-30-methyltritriacontane (V1) (-)-4-Methyltritriacontane

The formulas are written according to Fischer's convention. The relative configurations of the iodide (III) and the hydrocarbon (VI) follow from the alcohol (II) and the ketone (V) respectively, but, as Fischer's convention does not apply to iodides and hydrocarbons, the prefix has been omitted for III and VI.

The thermal and x-ray data for (-)-4-methyltritriacontane are given in Tables I and II, the corresponding data for phthiocerane and DL4-methyltritriacontane being included for comparison. The latter two were previously (1) found to possess two crystalline modifications, of which the one stable below 46° is identical with the orthorhombic (A) form of the normal chain hydrocarbons. The high temperature form, which is the

stable form between a temperature of 46° and the melting point, does not, however, correspond to the hexagonal high temperature form of the *n*-hydrocarbons, the crystal structure instead being one of lower symmetry (with tilted chains). The same crystalline forms are found for (-)4-methyltritriacontane, but the latter can also exist in a third form in which

1 nermai Data jor Hyarocaroons							
		Transition point			Solidi-	Remelting	
Compound	Crystals from On heating		On cooling	M.p. fication point		point	
		° C.	•C.	*C.	°C.	° C.	
Phthiocerane	Acetone	45.6-46.8	46	59.1-59.3	59.0	59.1-59.3	
DL-4-Methyltritri- acontane	Chloroform	45.6-46.4	46	58.6-58.7	58.5	58.6-58.7	
(-)-4-Methyltritri- acontane	Acetone Chloroform	Below 46	46* 46*	60.5-60.7† 61.6-61.8		58.5-58.6 58.5-58.6	

TABLE I
Thermal Data for Hydrocarbons

TABLE II
Crystal Data for Hydrocarbons

Crystal spacings

C form A form B form Compound Long Side Long Side Long Side spacing spacings spacing spacings spacing spacings A A A A 46.3 4.11, 3.70 42.4 4.36,4.03 Phthiocerane . . DL-4-Methyltritriacontane... 46.3 4.11, 3.70 42.2 4.36,3.97 (-)-4-Methyltritriacontane... 4.12, 3.72 42.0 4.34.4.03* 37.6 4.12.3.86. 46.23.64

the chains are still more tilted towards the basal plane than in the high temperature form of the pL-hydrocarbon. For convenience, the different crystal modifications are called A, B, and C respectively, in order of increasing tilt of the chains. On crystallization from acetone the optically active hydrocarbon is obtained in the A form. The appearance of the specimens and the diffraction patterns are identical with those of the A form of the pL-hydrocarbon. Only three 00l reflections are observed. The A form

^{*} After solidification of the melt.

[†] The melting points of specimens crystallized from acetone vary somewhat, probably owing to the simultaneous formation of varying small amounts of the B form in the transition from the A form into the C modification on heating.

^{*} At 55°.

may exist up to a temperature of 46°, at which point (or usually a few degrees lower) a transition occurs, mainly to the C form. Transition to the C form can also be induced at room temperature by mechanical treatment, i.e. pressing, of the specimen. The C form, which is stable up to its melting point at 61.6–61.8°, can be obtained from a slowly evaporating chloroform solution in the form of small lustrous plates. Under the microscope the latter are seen to be well developed single crystals, and pressed specimens give very good x-ray diffraction patterns showing many 00l reflections. The molten active hydrocarbon solidifies at 58.4° and remelting takes place at 58.5–58.6°, the form obtained on solidification evidently being identical with the high temperature form (B) of the pl-hydrocarbon. On cooling, the B form of the active hydrocarbon is transformed at about 46° to a mixture of the A and C modifications. On reheating a specimen from room temperature, melting occurred at 59–60°.

The pL-hydrocarbon on crystallization from chloroform is obtained in the A form. While the solvent rapidly evaporates from a chloroform solution of the active hydrocarbon, solutions containing the racemic hydrocarbon become covered with a thin transparent film which effectively retards further evaporation. Specimens of the pL-hydrocarbon obtained from acetene (A form) were previously reported (1) to give only three 00l reflections, but the films obtained on chloroform solutions give some weak but distinct higher order reflections, indicating a higher degree of regularity in the crystallographic direction within the films than in the pressed specimens.

The behavior of several mixtures containing the active and the pl-hydrocarbon in different proportions has been investigated. A mixture containing 15.2 per cent of the pl-hydrocarbon crystallized from chloroform in the C modification and melted at 59.9–60.2°. The solidification point was 58.3° and remelting occurred at 58.5°. A specimen containing 26.8 per cent of the pl-hydrocarbon crystallized under similar conditions was found to consist of a mixture of the A and C modifications. The melting point was 58.6–61°, and the solidification and remelting points were exactly the same as for the mixture containing 15.2 per cent of the pl-hydrocarbon. Finally, mixtures containing 52.8 and 67.1 per cent respectively of the pl-hydrocarbon were obtained from chloroform in the A modification and their thermal behavior was identical with that of pl-4-methyl-tritriacontane.

The results now described show that the high temperature (B) form of the racemic hydrocarbon is a racemic solid solution. They furthermore indicate that phthiocerane is a racemic or partially racemic hydrocarbon and that the difference in melting point between pl-4-methyltritriacontane and phthiocerane must be due to the presence of a small amount of a higher

homologue in the latter. Finally, the results suggest that phthiocerol has at least one of the functional groups at, or in the α position to, the carbon atom carrying the methyl side chain.

EXPERIMENTAL1

p(-)-21-Methyltetracosanol-1 (II)—The reduction was carried out in the manner described by Nystrom and Brown (9). p(-)-21-Methyltetracosanoic acid (8) (0.96 gm.) was dissolved in 50 ml. of dry ether. 15 ml. of a 10 per cent solution of lithium aluminum hydride (Metal Hydrides, Inc.) in ether was added and the mixture refluxed for 1 hour. Water was added dropwise until the evolution of gas ceased. Dilute sulfuric acid was then added until the white precipitate had dissolved. The mixture was transferred to a separatory funnel. The ether layer was washed twice with water and dried by means of sodium sulfate. The ether was removed by distillation, leaving a beautifully crystalline residue. Crystallization from light petroleum gave 0.76 gm. (82 per cent of the theoretical) of p(-)-21-methyltetracosanol-1 in the form of lustrous plates, m.p. 59.6–59.8°. Previously melted material showed the same melting point; long x-ray spacing (pressed specimen) 32.4 A. A melted specimen gave two sets of 00l reflections, corresponding to long spacings of 32.4 and 44.7 A respectively.

```
Optical Rotation—\alpha_{\rm D}^2 = -0.07^{\circ} (chloroform; l, 2 dm.; c, 3.8%); [\alpha]_{\rm D}^2 = -0.9^{\circ}; [M]_{\rm D}^2 = -3.2^{\circ}

Analysis—C<sub>24</sub>H<sub>50</sub>O (354.6). Calculated. C 81.44, H 14.22

Found. "81.21, "14.26
```

(-)-1-Iodo-21-methyltetracosane (III)—D(-)-21-Methyltetracosanol-1 (0.70 gm.), iodine (0.28 gm.), and red phosphorus (0.0198 gm.) were kept at a temperature of 145° on an oil bath for 4 hours. The reaction product was extracted with ether and the ether solution washed with 5 per cent sodium hydroxide solution, followed by water. After the solution had been dried with sodium sulfate, the solvent was removed by distillation and the residue crystallized from 99.5 per cent ethanol, giving 0.81 gm. (88 per cent of the theoretical) of white microcrystalline (-)-1-iodo-21-methyltetracosane. The melting point was 43.2-43.5°, unchanged after previous melting; long x-ray spacing (pressed or melted specimen) 36.4 A.

```
Optical Rotation—\alpha_{\rm D}^{\rm m} = -0.06^{\circ} (chloroform; l, 2 dm.; c, 4.2%); [\alpha]_{\rm D}^{\rm m} = -0.7^{\circ}; [M]_{\rm D}^{\rm m} = -3.3^{\circ}
```

Analysis—C₂₄H₄₉I (464.6). Calculated, I 26.52; found, I 26.59

D(-)-8-Keto-30-methyltritriacontane (V)—The active ketone was synthesized in the manner previously described for the racemic 4-methyl-substi-

¹ The thermal and x-ray investigations were carried out as described previously (1).

tuted isomer (1), with 0.76 gm. of (-)-1-iodo-21-methyltetracosane, 0.33 gm. of methyl 3-ketodecanoate (10), 2.5 ml. of methyl-n-propyl ketone, and 0.8 gm. of potassium carbonate. The mixture was boiled under a reflux for 22 hours. The alkylation product was isolated and subjected to hydrolysis and ketonic cleavage by dissolving in a solution of potassium hydroxide (3 gm.), water (3 ml.), and methanol (45 ml.). The mixture was kept at a temperature of 50° for 30 hours. Part of the ketone formed separated in the hydrolysis mixture in the form of small lustrous plates. These were filtered off and found to weigh 0.18 gm. The filtrate was worked up in the ordinary manner, and crystallization from acetone gave 0.25 gm. of ketone. Total yield, 0.43 gm. or 52 per cent of the theoretical. The specimen of p(-)-8-keto-30-methyltritriacontane recovered from the hydrolysis mixture melted at 65.0-65.2°, solidified at 56.9°, and remelted at 57.1°. The low melting form is stable only near the melting point; melted material cooled to room temperature was found to possess the higher melting point. The material crystallized from acetone melted at 64.8-65.2°; long x-ray spacing (pressed specimen) 37.6 A, calculated from a very good photograph. A specimen prepared by means of the hot wire technique gave a long spacing of 46 A (vertical form), but the diffraction pattern also showed some faint lines corresponding to the 37.6 A spacing.

```
Optical Rotation—\alpha_{\rm D}^{\rm n}=-0.04^{\circ} (chloroform; l,\ 1\ \rm dm.;\ c,\ 5.5\%);\ [\alpha]_{\rm D}^{\rm n}=-0.7^{\circ};\ [M]_{\rm D}^{\rm n}=-3.6^{\circ}\sim-4^{\circ}
Analysis—C<sub>34</sub>H<sub>88</sub>O (492.9). Calculated. C 82.85, H 13.91
Found. "82.83, "13.87
```

(-)-4-Methyltritriacontane (VI)—The ketone just described (0.32 gm.) was reduced by Clemmensen's method (cf. (1)). 6 gm. of amalgamated zinc, 6 ml. of concentrated hydrochloric acid, and 2 ml. of glacial acetic acid were used initially. The zinc, hydrochloric acid, and glacial acetic acid were replaced by fresh reagents at 8 hour intervals. After a total of 80 hours the hydrocarbon was isolated and crystallized from acetone, giving 0.25 gm. of white microcrystalline material melting at 58–60° and solidifying at 57.6°. One further crystallization from acetone gave 0.22 gm. melting at 60–60.4°. The material solidified at 58.2° and remelted at 58.5°. 0.13 gm. of this material was treated with concentrated sulfuric acid at 120° for a period of 30 minutes. The recovered hydrocarbon melted, after crystallization from acetone, at 60.5–60.7°, solidified at 58.4°, and remelted at 58.5–58.6°. A detailed description of the thermal and crystal behavior of this material has already been given in the introduction.

```
Optical Rotation—\alpha_{\rm D}^{2} = -0.03^{\circ} (chloroform; l, 1 dm. (micro tube); c, 4.8%); [\alpha]_{\rm D}^{12} = -0.6^{\circ}; [{\rm M}]_{\rm D}^{12} = -3^{\circ}

Analysis—C_{34}H_{70} (478.9). Calculated. C 85.27, H 14.73

Found. "85.10, "14.68
```

We are indebted to Mr. W. Kirsten and Mr. I Alperowicz for the microanalyses, and to the National Swedish Antituberculosis Association and the Rockefeller Foundation for financial support.

SUMMARY

The levorotatory enanthiomorph of 4-methyltritriacontane has been synthesized and its thermal behavior and crystal structure compared with those of DL-4-methyltritriacontane and phthiocerane. The optically active hydrocarbon has three different crystalline modifications, two of which are identical with the high and low temperature forms respectively of the DL-hydrocarbon and of phthiocerane. It is shown that the high temperature form of DL-4-methyltritriacontane is a racemic solid solution and that phthiocerane is a racemic, or partially racemic, hydrocarbon. The results indicate that the difference in melting point between the DL-hydrocarbon and phthiocerane is due to the presence of a small amount of a higher homologue in the latter, and that phthiocerol has at least one of the functional groups situated at, or in α position to, the carbon atom carrying the methyl side chain.

BIBLIOGRAPHY

- 1. Ställberg-Stenhagen, S., and Stenhagen, E., J. Biol. Chem., 173, 383 (1948).
- Ställberg-Stenhagen, S., Stenhagen, E., Sutherland, G. B. B. M., Sheppard, N., and Walsh, A., Nature, 160, 580 (1947).
- 3. Stodola, F. H., and Anderson, R. J., J. Biol. Chem., 114, 467 (1936).
- 4. Reeves, R. E., and Anderson, R. J., J. Biol. Chem., 119, 535 (1937).
- 5. Cason, J., and Anderson, R. J., J. Biol. Chem., 119, 549 (1937).
- 6. Cason, J., and Anderson, R. J., J. Biol. Chem., 126, 527 (1938).
- 7. Ginger, L. G., and Anderson, R. J., J. Biol. Chem., 157, 213 (1945).
- 8. Ställberg-Stenhagen, S., Ark. Kemi., in press.
- 9. Nystrom, R. F., and Brown, W. G., J. Am. Chem. Soc., 69, 2548 (1947).
- 10. Ställberg-Stenhagen, S., Ark. Kemi, Mineral. o. Geol., 20 A, No. 19 (1945).

METABOLISM OF TESTOSTERONE BY LIVERS OF DIFFERENT SPECIES OF ANIMALS*

By LEO T. SAMUELS, MAX L. SWEAT, BLAINE H. LEVEDAHL, M. M. POTTNER, AND M. L. HELMREICH

(From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City)

(Received for publication, August 1, 1949)

In previous papers (1-4) it has been demonstrated that the metabolism of testosterone by liver tissue involves at least two enzyme systems, one requiring diphosphopyridine nucleotide (DPN) and the other citrate as cofactor. The former compound is involved in the oxidation of the alcohol group on C-17 to a ketone; the latter increases the rate of destruction of the α,β conjugation in Ring A formed by the Δ_4 double bond and the ketone group on C-3. The variation in testosterone metabolism has been investigated in a wider series of species, and evidence of a phylogenetic development of the enzyme systems has been found.

Methods

Except in the case of the steer and the human, liver tissue was removed in the laboratory immediately after death by decapitation. Human liver tissue was removed during surgery and immediately immersed in ice-cold Locke's solution. It was then transported to the laboratory as rapidly as possible. Steer liver was obtained at the slaughter-house, cooled, and also brought to the laboratory within a short time. The tissue was immediately sliced with a razor or minced with a mechanism made up of multiple razor blades. It was then suspended in the buffer solution of testosterone and incubated in an atmosphere of oxygen, as previously described. Enzymic action was stopped by boiling the contents of the flask under a reflux for 20 minutes, after which extraction and analysis were carried out as in previous studies (5).

60 per cent DPN was procured from the Schwarz Laboratories. It was added directly to the flasks to give a final concentration of 0.001 m DPN. Sodium citrate was used at a concentration of 0.001 m.

The concentration of molecules containing the α,β -unsaturated ketone group in Ring A was estimated by the ultraviolet absorpton band at 240

*This investigation was supported by grants from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, the American Cancer Society through the Committee on Growth of the National Research Council, and Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

TAB: y L

(40 - 50)92) (17-76)1015) (236-292) (98-124)(63 - 66)y per 8m. per hr. 17-Keto-steroids formed Buffer + DPN + citrate (42- 9 143) 240) 362) 98 y per 8m. per hr. (>358->367) (>252->294) α,β destruction 106 (174-(352-> 365 88 53) 42) (580-666) (107-167) r per gm. 17-Keto-steroids formed (21-(14-(31-Buffer + citrate (13- $\frac{33}{2}$ (14-62)(37-81) (92 (88-108) (303-304)(144-216)(296-351)(93-100) (250-259) (166-197)6 a, B de-struction <u>8</u> 62 S_{p} (87 - 97)(312-359)(308-306)(29 106-107) y per gm per hr 17-Keto. steroids formed (62-0 8 DiffBuffer + DPN 167) 588) y per gm per hr. 8 266) (>365->367)202 38 68 162) a, & destruction -845485-(145ę $\frac{150}{1}$ 8 8 (86-105)350-406) (163-211) 33 8 y per am. per hr. 17-Keto-steroids formed (18-Buffer only α,β destruction (16-42)(170-193)y per km. (54-69)(26-62)(0-227)96-100(39-54)201-204) 178-212) 380 195 181 Original testoster-one con-centra-tion Destru y per 200 82 8 8 576 8 8 88 88 8 888 No. of t livers combined 9 n ~ 20 O က Fish " fasted 48 hrs.† Animal Rattlesnake. Chicken . . Rat fedt... ъ fed, ♀ Steer Turtle Rabbit ï Frog. z ×

Guinea nie	6	000	90	101	00,		78.	44.	3	100		2
· · · · · · · · · · · · · · · · · · ·	•	8	3		8		134	3	7	107		70
1			(52-149)	(98-111)	150	179)	(102-174)	(152-160)	(24-33)	(256 -	298	(36-65)
Dog, o	_	88	91	31	154		100	117	31	246		4 0
,			(78-104)	(28-37)	(138	164)	(97-104)	(104-127)	(28-34)	-682)	254)	(44-52)
Monkey	_	288	155	133	210		170	284	122	202		101
			(146-162)	(146-162) (122-148)	88	(617	(147-190)	(282-281)	- 219) (147–190) (282–287) (118–128) ((284 1	306	(90-112)
Human	9	200	137	25	164		153					
			(106-196)	(40-68)								
	-	88	224	132	183		270	291	32	312		224
			(223-226)	(118-145)					(86-104)	906)	317)	(206-242)
3	_	883	305	8			183					
			(294-316)	(66- 94)	-986	547)	547) (180-186)	***************************************				

Except in the case of the fish and turtle, in which data from three incubations are combined in each case, each line represents a single incubation. The number of livers in all cases except the two mentioned represents the numbers which were minced together to furnish sufficient sample for the series. The numbers in parentheses indicate the range of values obtained from individual incubation flasks. Each flask contained 25 ml. of buffer and 0.50 to 1.00 gm. of liver mince. In most cases the results from three flasks have been averaged to give the mean value. Testosterone concentration is given because, over the range involved, destruction varies almost directly as substrate concentration. This must be considered in comparing the rates of destruction

* Micrograms of steroid per gm. of liver mince per hour. † These rats were from the same litters.

mμ. The 17-ketone group was determined by the absolute alcohol method of Callow, Callow, and Emmens (6). Spectrophotometric readings were made at 440, 520, and 600 mμ wave-lengths. These were corrected for background absorption on the assumption that it showed a constant fall over this range. When the three wave-lengths mentioned were used, the brown color of testosterone in the quantities employed gave no significant value after correction.

Results

The results of studies of the various species are summarized in Table I. The livers of fish, amphibians, and reptiles all destroyed the α, β structure in testosterone at a slow rate, but no evidence of oxidation at C-17 was found. The addition of either DPN or citrate did not affect the result significantly. The enzyme system involved, therefore, apparently does not require these compounds as cofactors.

A typical series of experiments on one form of reptile, the turtle, is shown in Table II. While the activity of slices was approximately twice that of the minced tissue from any one individual, the reaction to the addition of cofactors was the same. There did not seem to be any acceleration in the presence of DPN or citrate. The variation between turtles of the same sex was as great as that between turtles of opposite sexes.

With birds and mammals, however, enzyme systems requiring each of these cofactors appear. If DPN was added to minces or slices of livers from members of these orders, 17-ketosteroids were formed in all animals except the rabbit. In the latter, 17-ketosteroids were produced in the presence of the liver tissue but the nucleotide seemed to have no effect on this process.

In addition to the effect on C-17, the presence of DPN increased the destruction of the conjugated system in Ring A as measured by the decrease in absorption at 240 m μ , again with the exception of the rabbit. This would appear to be due to the ability of an enzyme to reduce the ketonic group on C-3 more readily when there is a ketone rather than an alcohol on C-17. When the 17-ketosteroids formed by chicken liver, which has a relatively high concentration of this oxidative system, were treated with succinic anhydride, all were found in the water-soluble fraction. They must, therefore, have had an alcohol group as well as the 17-ketone. The most likely assumption is that the ketone on C-3 was reduced to an alcohol and formed Δ_t -androstenol-3-one-17.

As can be seen in Table I, in all species except the steer the number of conjugated groups disappearing equals or exceeds the number of ketonic groups formed at the other end of the molecule. This was true both before and after the addition of exogenous DPN. The problem of the rôle

of the 17-ketosteroids has been analyzed in the case of chicken liver. This tissue forms relatively large amounts of alcoholic 17-ketosteroids from testosterone without added cofactor.

In Table III the effect of time on the proportions of the different fractions is demonstrated. The non-conjugated non-17-ketosteroids represent the difference between Columns 1 and 2. When incubation time was increased, the concentration of the 17-ketosteroids remained relatively constant but the amount in Column 3 increased. These observations could

Table II

Metabolism of Testosterone by Turtle Liver
The change is given as micrograms per gm. per hour.

Temperature	No. and sex	Preparation	Buffer only*	Buffer + 0.001 m DPN	Buffer + 0.001 m citrate	Buffer + 0.001 m DPN + 0.001 m citrate
•			α,β groups destroyed	α,β groups destroyed	α,β groups destroyed	α,β groups destroyed
°C.						
30	1 🗗	Mince	42	38	42	
30	1 🗗	Slices	61		66	1
30	2 ♂	Mince	16		14	14
30	2 ਨਾ	Slices	70		59	
38.5	3 ♂	Mince	21	30	29	
30	4 ♀	"	40	38	62	
30	4 ♀	Slices	95		85	I
30	5 ♀	Mince	34		57	
Average .		Mince	31	35	41	
"		Slices	75		70	

^{*} The buffer solution contained KCl 0.0056 M, MgCl₂ 0.0021 M, NaCl 0.08 M, Na₂HPO₄-NaH₂PO₄ buffer (pH 7.4) 0.04 M, glucose 0.1 per cent. Approximately 1 gm. of mince or slices was used per flask together with 200 γ of testosterone and 25 ml. of buffer solution. The extracts from all incubations were analyzed for 17-ketosteroids but none were found.

best be explained if the alcoholic 17-ketosteroids were themselves intermediates which underwent a reduction at C-17.

The sequential relationship of the formation of 17-ketosteroids and their breakdown was investigated by comparing the effect of chicken and rat livers. In the absence of added DPN rat livers form little 17-ketosteroids but break down many more α, β groups. Testosterone was first incubated with mince from one species of liver and the products were then incubated with liver mince of the other species. Analyses for α, β conjugation and 17-ketosteroids were made at the end of each incubation. The

results are shown in Table IV.' It is evident that bird liver was unable to form 17-ketosteroids from the products of the action of rat liver on testosterone. The small amounts present could be accounted for by conversion of the residual testosterone. When the order was reversed, how-

Table III

Effect of Incubation with Chicken Liver Mince on Different Parts of Testosterone
Molecule

The tissue was incubated with 400 γ of testosterone in glucose buffer.

Flask No.	Amount of liver	Incubation time	α,β groups destroyed (1)	Alcoholic 17-ketosteroids formed (2)	Non-conjugated non-17-keto- steroids formed (3)
:	gm.	min.	per ceni	per ceni	per cent
1	0.975	60	53	39	14
2	0.990	60	62	35	27
3	1.180	120	80	39	41
4	1.110	120	87	44	43

Table IV

Balance Sheet for Reincubation Experiment in Which Both Rat and Chicken Livers

Were Used in Series

The 17-ketosteroids formed by ch	hicken liver were	destroyed by	v rat liver.
----------------------------------	-------------------	--------------	--------------

Incu- bation		Rat liver by chick	followed ten liver	followe	en liver d by rat ver
		7	γ	γ	γ
1st	Testosterone added	150	150	150	150
	" recovered	64	60	10	13
	" destroyed	86	90	140	137
	17-Ketosteroids formed	0	0	107	104
2nd	Testosterone present, start	64	60	10	13
	17-Ketosteroids present, start	0	0	107	104
	Testosterone recovered	37	38	0	0
	17-Ketosteroids recovered	24	23	40	40
	Testosterone destroyed	27	22	10	13
	17-Ketosteroids destroyed			67	64
	" formed	24	23		

ever, rat liver was able to convert the 17-ketosteroids formed by the bird liver into non-ketonic unconjugated compounds. This would seem to confirm the previous evidence that the formation of 17-ketosteroids might be an intermediate step.

When citrate was added to any of the tissue preparations of birds or mammals, there was a significant, often a marked, increase in the destruc-

tion of the α,β structure. As shown previously (3), this appears to be a specific effect of citrate. There was no effect in the other orders of animals.

In addition, there was usually a definite decrease in 17-ketosteroid formation if these would otherwise have been formed. One exception was the monkey, in which there was little effect on the 17-ketosteroids but a marked increase in α, β bonds destroyed. This second effect of citrate appears to be unrelated to the first, as can be seen in Table I.

A comparison of the results when both citrate and DPN were added with those when either was used alone indicates that the effects were roughly the algebraic sum of the individual influences. The destruction of α,β groups was greater than with either alone, while the concentration of 17-ketosteroids was intermediate. Within mammalian species themselves there was considerable variation in the activity of the different enzyme systems.

The liver from only one herbivore was investigated, that of the steer. In Table I it will be seen that there was little or no destruction of the conjugated system or oxidation at C-17 by the mince alone. The addition of DPN led to a marked formation of 17-ketosteroids but no significant change in Ring A. Addition of citrate brought about the change in α, β structure but no oxidation at the other end of the molecule. If both cofactors were added, changes occurred in both positions. The relative inactivity of steer liver minces and extracts, together with the apparently single action in the presence of each cofactor alone, has been repeatedly observed. This would seem to indicate either a low level of these cofactors in the original tissue or a very rapid destruction during preparation. Apparently the enzyme system which does not require either of these cofactors and which is present in the livers of the lower orders is not found in steer liver, or else some as yet unknown cofactor is absent.

Rabbit liver seems to show a more fundamental difference from the liver of other mammals. While the unsupplemented liver both destroyed the α,β structure and formed 17-ketosteroids, the addition of DPN had no influence on the rate of either reaction. Citrate increased the destruction of α,β groups and depressed the formation of 17-ketosteroids, but the simultaneous addition of DPN had no significant additional effect. These observations would all be true if triphosphopyridine nucleotide rather than DPN were the cofactor in this system. The definitive experiment with added triphosphopyridine nucleotide has not yet been done.

DISCUSSION

The evidence here presented would indicate that, associated with the development of homeothermic control, there was an increase in the complexity of the enzyme systems degrading the gonadal steroids.

In the poikilotherms body temperature is ordinarily lower than that of the homeotherms and chemical reactions, both synthetic and degradative, would be slower in the presence of the same concentrations of substrate and catalyst. The turn-over of such active compounds as the sex steroids would therefore be less, and the need for complex and rapid adjustment rare. Moreover, the changes in temperature of the organism due to season and environment themselves regulate the production of hormones. A simple system of inactivation would be sufficient.

In the homeotherms, however, production of sex steroids is both more rapid and more complex. Cyclic phenomena occur under conditions of a constant internal physical environment. In turn more rapid inactivation mechanisms have been developed. These, however, require labile energy transfer systems. Thus, while destruction may be more rapid, it also becomes subject to the supply of these systems. A more rapid, more adaptable, but also more labile system has thus evolved.

SUMMARY

The ability of liver minces and slices from various species and orders of animals to destroy testosterone has been investigated, both in the absence and in the presence of added diphosphopyridine nucleotide and citrate.

In fish, amphibians, and reptiles there appears to be an enzyme system which slowly destroys the conjugated double bond system in Ring A but does not form 17-ketosteroids. No system, the activity of which was increased by DPN or citrate, was identified.

In birds and mammals, systems requiring DPN and citrate are present. The DPN-catalyzed reaction leads to the formation of 17-ketosteroids, while that requiring citrate acts on the α, β conjugation in Ring A without forming 17-ketosteroids. The formation of 17-ketosteroids in the presence of DPN is also decreased by citrate.

The relative concentrations of the two systems requiring cofactors, as well as cofactor availability, vary between different species. The chicken appears to have a relatively high concentration of the DPN-activated system, man and the guinea pig an intermediate balance, and the rat and dog a relatively high activity of the citrate-activated enzyme. Steer liver shows little activity unless cofactors are added, but destruction occurs in the presence of either of the two compounds studied which play this rôle. This would indicate a relative unavailability of these substances, at least under the conditions of preparation of the tissue. Rabbit tissue seems to use some cofactor other than DPN in the oxidation of C-17.

The relation of the development of the systems activated by DPN

and citrate to the appearance of homeothermic mechanisms is discussed. A tentative outline of the possible order of enzymic reactions is also given.

BIBLIOGRAPHY

- 1. Samuels, L. T., McCaulay, C., and Sellers, D. M., J. Biol. Chem., 168, 477 (1947).
- 2. Sweat, M. L., and Samuels, L. T., J. Biol. Chem., 173, 433 (1948).
- 3. Sweat, M. L., and Samuels, L. T., J. Biol. Chem., 175, 1 (1948).
- 4. Samuels, L. T., and Pottner, M., Federation Proc., 6, 287 (1947).
- 5. Samuels, L. T., J. Biol. Chem., 168, 471 (1947).
- 6. Callow, N. H., Callow, R. K., and Emmens, C. W., Biochem, J., 32, 1312 (1938).

THE SEDIMENTATION BEHAVIOR OF BOVINE AND EQUINE IMMUNE PROTEINS*

By EMIL L. SMITH AND DOUGLAS M. BROWN

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City)

(Received for publication, October 15, 1949)

In previous studies, we have described the isolation, by chemical methods, and some chemical and physical properties of immune proteins from the cow and the horse (1-4). In extension of this work, the sedimentation behavior of these same preparations has been studied in the ultracentrifuge. It was found that the principal component of these proteins has a sedimentation constant of about 7 Svedberg units and in association with this there regularly occurs another component of about 10 Svedberg units. While most of our observations are in good accord with those of previous investigators, some minor differences have been found and these will be discussed following the presentation of our data.

Methods and Materials

The sedimentation studies were made in the Spinco² electrically driven ultracentrifuge. A brief description of this instrument, and the controls and procedures as used in this laboratory, has been given in a previous publication (5). All of the runs were made at 59,780 R.P.M., equivalent to centrifugal fields of $240,000 \times g$ and $300,000 \times g$ at the meniscus and base.

The isolation of the bovine proteins of plasma, colostrum, and milk has been described (1, 2). Preparation A of bovine γ -globulin was derived from normal steer blood obtained at the slaughter-house. Preparation B was isolated from the plasma of cows hyperimmunized with a mixture of antigens: diphtheria toxin (or toxoid), vaccinia virus, and a killed culture of *Hemophilus pertussis*; the colostrum, milk, and T-globulins were from the same animals (6).

The immune globulins of the horse used in this study are those previously isolated by Smith and Gerlough (4), and contained high titers of tetanus

- This investigation was aided by a grant from the United States Public Health Service.
- ¹ The Svedberg unit is defined as 1×10^{-13} sq. cm. per second, and is abbreviated as S.
 - ² Specialized Instruments Corporation, Belmont, California.

antitoxic activity. All of the preparations used in this study had been dried from the frozen state and were studied after standing in dry form for 1 to 3 years. Samples which were salt-free were dissolved in the appropriate solvent immediately before use; others were dialyzed at 2° until they were in equilibrium with the solvent.

Results

Bovine Proteins—Measurements on these proteins are presented in Table I. The sedimentation constants were found to be essentially independent of the protein concentration within the limits studied. It is apparent that none of these proteins is molecularly monodisperse, and that they are all very similar in composition. In each instance, the main component possesses a sedimentation constant $(s_{20, w})$ near 7 Svedberg units and represents 80 to 90 per cent of the total refractive increment. This is always accompanied by smaller amounts of a second component which has a value of about 10 S; this second particle size represents about 10 to 20 per cent of the total sedimenting area. Two of our preparations also show the presence of additional substances. The colostrum pseudoglobulin contains about 10 per cent of slowly sedimenting material (about 2 S). The colostrum euglobulin contains about 5 per cent of heavy material which is detectable only at higher protein concentrations.

Hansen, Potter, and Phillips (7) reported that colostrum pseudoglobulin isolated by the method of Smith (1) showed only a single sedimenting boundary with a value of $s_{20, w} = 7$ S. This differed from the behavior of such material previously reported by one of us in preliminary observations (2), and from that observed in the present study. At least two components were always observed in our pseudoglobulin preparations obtained from either milk or colostrum. Dr. R. G. Hansen (now of the University of Utah) kindly gave us a dried sample of colostrum pseudoglobulin which was prepared by him at the University of Wisconsin by the same method which we used (1). On examination in the ultracentrifuge, there was no doubt of the presence of two components, although the second one was present in smaller amounts than in our preparations. A sedimentation diagram of Hansen's preparation is shown in Fig. 1, A in comparison with our colostrum and milk pseudoglobulins (Fig. 1, B and C). The milk and colostrum euglobulins are shown in Fig. 1, D and E.

Our preparations of the bovine plasma globulins (γ and T) likewise contain two components, in agreement with earlier observations (2). However, Hess and Deutsch (8) reported that they obtained from normal cows a γ_2 -globulin (equivalent in electrophoretic mobility to our γ -globulin) which was essentially monodisperse (s_{20} , w = 7.4 S), while their γ_1 -globulin (equivalent to our T-globulin) contained about 4 per cent of heavier con-

TABLE I
Sedimentation Behavior of Bovine Immune Proteins

The values of the sedimentation constants $(S_1, S_2, S_3, \text{ and } S_4)$ are for $s_{20, w}$ in Svedberg units and incorporate the usual corrections for the viscosity and density of the medium. The temperature is the average for the period during the run when photographs were being taken. The parenthetical values give the amount of each component as estimated from its percentage of the total sedimenting area. All of these runs were performed with 0.15 m sodium chloride as the solvent.

Protein	pН	Pro- tein con- centra- tion	Tem- pera- ture	Si	S ₂	Sa	<i>S</i> 4
		per cent	•c.	Mercina Production States States			
Milk pseudo-	6.7	1.1	25.2		6.8 (85%)	8.9 (15%)	
globulin							
<i>(1 (</i> 6	6.2	1.0	30.0		6.8 (87%)	9.5 (13%)	
Colostrum pseu-	6.8	0.25	23.7	2.0 (9%)	6.8 (77%)	10.0 (15%)	
do globul in							
ű ű	5.8	0.5	25.5	1.4 (11%)	6.8 (77%)	10.0 (12%)	
11	5.8	1.0	23.9	2.1 (11%)	6.9 (78%)	10.5 (11%)	
· · · · · · · · · · · · · · · · · · ·	6.6	1.0	23.6		7.4 (91%)	10.0 (9%)	
Milk euglobulin	6.2	1.2	23.7		6.8 (86%)	9.7 (14%)	
	6.2	0.9	26.7			10.8 (18%)	
Colostrum eu-	6.2	0.25	25.3			10.2 (22%)	
globulin		1 1		1			
"	6.1	0.5	23.4]	7.2 (80%)	10.4 (20%)	
	6.1	1.0	24.1		6.8 (77%)		17.6 (5%)
Plasma T-globu-	6.0	0.25	24.8		6.9 (80%)		, , ,
lin		! '			1		
	6.0	0.5	22.5		6.9 (89%)	9.7 (20%)	
	6.2	1.0	23.1		6.9 (86%)		
Plasma y1-globu-	7.0	1.1	23.2		6.8 (91%)		
lint				1	, , , ,	(1,0)	
Plasma y-globu-	6.7	0.25	18.7		6.9 (79%)	10.8 (21%)	
lin A		0.20	2000		(12,0)	(==,0,	
" "	6.7	0.5	23.9		7.0 (81%)	9.5 (19%)	
"	6.7	1.0	22.7			10.5 (16%)	
Plasma y2-globu-	6.6	1.0	24.0			10.2 (15%)	
lint	0.0	1.0	21.0		1.0 ((2)0)	10.2 (10/0)	
Plasma · γ-globu-	6.2	0.25	24.8		6.9 (80%)	9.9 (20%)	
lin B	0.2	0.20	44.0		0.0 (00/6)	0.0 (20/0)	
111 15	6.2	0.5	22.5		6.9 (89%)	9.7 (11%)	
"	6.2	1.0	23.1		6.9 (86%)	9.5 (14%)	
	0.2	1.0	40.1	1	0.8 (80%)	3.0 (12%)	

^{*} This sample was prepared from a normal animal by Dr. R. G. Hansen.

[†] These samples were provided by Dr. H. F. Deutsch of the University of Wisconsin. In his nomenclature, these samples, identified as γ_1 - and γ_2 -globulins, are equivalent to our T- and γ_2 -globulins respectively.

stituents with $s_{20, w} = 9$ S. Later, these investigators (9) obtained similar results with globulins prepared from hyperimmunized animals. Examination of samples kindly furnished to us by Dr. Deutsch indicates that the γ_1 preparation contains 9 per cent of the heavier component, and that the γ_2 preparation contains 16 per cent of the heavier component. Sedimentation patterns of the preparations made by Hess and Deutsch are shown in Fig. 1, F and G in comparison with ours (Fig. 1, H and I).

It must be noted that Hess and Deutsch studied their preparations immediately after fractionation, while our studies of their material were performed on dried preparations which had been kept by us for about a year or longer.

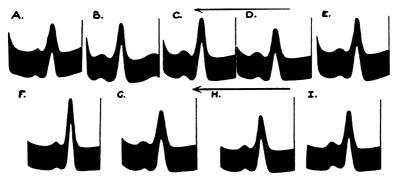


Fig. 1. Sedimentation patterns obtained on various bovine immune proteins A is a colostrum pseudoglobulin (Hansen) at 1.0 per cent; B is colostrum pseudoglobulin at 1.0 per cent; C is a milk pseudoglobulin at 1.1 per cent; C and C are milk (1.2 per cent) and colostrum (1.0 per cent) euglobulins (the colostrum euglobulin pattern does not show the heaviest component, C = 17.6 S); C and C are C1- and C2- globulins prepared by Hess and Deutsch (8); C3- C4 and C5- C5 are C5- C6- C6- C7- and C7- and C8- C9- C

All of the results discussed thus far were obtained on preparations isolated by chemical procedures. Recently, the separation of bovine serum proteins was accomplished by an electrophoresis-convection method (10). It was of considerable interest to examine the sedimentation behavior of some of these proteins. Dr. Cann, Dr. Brown, and Dr. Kirkwood very kindly permitted us to study two of their bovine γ -globulin preparations: Fraction E possessing an electrophoretic mobility of -1.24×10^{-5} sq. cm. per volt per second (in veronal buffer at pH 8.7) and Fraction G with a mobility of -2.25×10^{-5} . Their respective isoelectric points are 7.31 and 5.74; these preparations appear to correspond to γ -globulin (γ_2) and T-globulin (γ_1). Samples of these were furnished in lyophilized form and were dialyzed against 0.15 m sodium chloride.

Results on these preparations are given in Table II and representative

diagrams are shown in Fig. 2. It is evident that the results on these fractions are similar to those obtained on the chemically isolated ones in that the same two components are present: a large one with an s_{20} , w of about 7 S and a secondary one of about 10 S. However, it should be noted that the main component of Fraction E shows a pronounced concentration effect leading to an extrapolated value at zero concentration of 7.3 S.

Equine Proteins—Table III gives the results obtained in the ultracentrifuge on the tetanus antitoxin preparations. The γ - and T-globulins show results very similar to those obtained with the bovine proteins in that two

TABLE II

Sedimentation of Bovine γ -Globulins Prepared by Electrophoresis-Convection

The sedimentation constants are given in Svedberg units. The solvent was 0.15

M sodium chloride. Fraction G corresponds to γ_1 - or T-globulin and Fraction E to γ - or γ_2 -globulin.

Fraction	pН	Protein con- centration	Temperature	S_1	S ₂
		per cent	*C.		
G	8.5	0.25	28.9	6.7 (86%)	9.8 (14%)
	8.5	0.5	29.7	6.8 (84%)	10.6 (16%)
	8.5	1.0	27.3	6.7 (91%)	9.6 (9%)
${f E}$	8.0	0.25	28.8	7.1 (91%)	9.8 (9%)
	7.6	0.5	30.5	6.9 (94%)	10.5 (6%)
	8.0	0.8	29.2	6.7 (90%)	10.0 (10%)
	8.0	0.8	29.4	6.7 (90%)	10.0 10%)

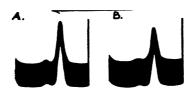


Fig. 2. Sedimentation patterns of bovine γ -globulins prepared by electrophoresis-convection (10). A is Fraction E at 0.8 per cent, and B is Fraction G at 1.0 per cent.

components were found and these possess similar sedimentation constants. The equine γ -globulin, like most of the bovine globulins, shows little or no effect of protein concentration on the sedimentation constants of the various components. In contrast to these, the equine T-globulin does show a marked concentration effect. The sedimentation constants found on extrapolation to zero concentration are 7.3 S for the main component and 10.3 S for the secondary component. This suggests that the components of the T-globulin may be much more asymmetrical than those of the γ -globulin.

It was of considerable interest to examine Fraction IV-C2, since previous study (4) had indicated that this pseudoglobulin fraction was higher in tetanus antitoxic activity than the γ - and T-globulins obtained from the

Table III
Sedimentation Behavior of Equine Immune Proteins

The conditions were the same as those given in Table I. All of the measurements were made with 0.15 m sodium chloride except those marked with an asterisk, which were performed with 0.1 m veronal buffer. The sedimentation constants are in Svedberg units.

Protein	рН	Protein concen- tration	Tempera- ture	S_1	S ₂	Sa
Market - with the second secon		per cent	℃ .			-
T-Globulin	6.2	0.25	24.0		6.8 (78%)	9.9 (22%)
	5.8	0.25	24.0		7.2 (77%)	10.2 (23%)
	6.1	0.5	24.4	1	6.9 (81%)	10.3 (19%)
***************************************	8.5	0.9	22.3	1	6.7 (88%)	9.4 (12%)
	5.5	1.0	22.4		6.6 (81%)	9.8 (19%)
γ-Globulin	5 .8	0.25	24.4		6.6 (88%)	8.4 (12%)
"	5.8	0.5	24.4	1	6.7 (87%)	9.0 (13%)†
** *	8.5	0.7	21.7	;	6.5 (91%)	9.9 (9%)
	5.0	1.0	26.5		6.5 (93%)	8.8 (7%)
	5.2	1.0	26.6		6.8 (93%)	9.2 (7%)
Globulin‡	5.7	0.5	24.9	6.2 (31%)	7.5 (60%)	14.8 (9%)
	5.6	0.5	28.4	5.6 (40%)	7.6 (53%)	15.0 (7%)
" *	8.5	0.9	24.3	4.8 (24%)	6.9 (68%)	11.4 (8%)
	8.8	1.0	24.3	6.0 (27%)	7.1 (62%)	8.4 (11%)
"	5.3	1.2	26.1	5.3 (32%)	6.5 (59%)	7.9 (9%)

[†] This component showed some evidence of splitting into two.

[†] Fraction IV-C2.



Fig. 3. Sedimentation behavior of equine immune fractions. A is a T-globulin at 1.0 per cent concentration, B is a γ -globulin at 1.0 per cent, and C is Fraction IV-C2 at 1.2 per cent.

same plasma. Electrophoretically, Fraction IV-C2 contains no γ -globulin and only about 1 per cent T-globulin and is mainly a mixture of β - and α -globulins with peak mobilities between 3.0 and 4.9 \times 10⁻⁵ sq. cm. per volt per second in veronal buffer at pH 8.5. In the ultracentrifuge,

Fraction IV-C2 contains three well defined components, the main one having a value of $s_{20, w} = 8.2$ S when extrapolated to zero concentration. The heaviest component also shows a significant variation of $s_{20, w}$ with concentration; at zero concentration $s_{20, w} = 20$ S. The lightest component shows considerable variability both in amount and sedimentation constant and shows some evidence of being a mixture. Representative sedimentation diagrams of γ - and T-globulins and Fraction IV-C2 are shown in Fig. 3.

It should be recalled that some investigators (11, 12) have observed the presence of components with $s_{20, w} = 20$ S in certain antibody preparations obtained from cow and horse. Such heavy components have also been found in human γ -globulin preparations (13, 14). In this investigation, most of the preparations did not contain such heavy components and similar findings have also been reported earlier (2, 8, 9, 15). The only samples which do contain such components are euglobulin from colostrum and the equine Fraction IV-C2. However, there is no evidence as yet that antibody activity is associated with these heavy components present in our preparations.

Deutsch and Nichol (15) have also prepared γ -globulins from normal and hyperimmune horse sera by chemical fractionation. Their sedimentation studies gave results very similar to those reported here; 80 per cent of the globulin had a sedimentation constant of 6.8 to 7.2 S and about 20 per cent sedimented in the range of 8 to 15 S.

DISCUSSION

Some earlier studies have been made on the sedimentation behavior of immune fractions obtained by precipitation with specific antigens. Kabat (11, 12) found that antipneumococcus antibodies of the cow and horse are mainly heavy molecules with $s_{20, w} =$ about 20 S, and a molecular weight of about 1 million. In contrast, it has been found that undigested diphtheria antitoxin of the horse has a sedimentation constant near 7 S and a molecular weight of 160,000 to 180,000 (16-18). Thus, the main component of the γ and T preparations of tetanus antitoxin studied in this investigation is similar to the diphtheria antitoxin. The observations of Deutsch and Nichol (15) on globulins containing both tetanus and diphtheria antitoxins are in agreement with these studies. It is likely that the antitoxic component of our Fraction IV-C2 is also of about the same size.

Most of the bovine globulins studied in this investigation do not contain detectable amounts of heavy molecules with $s_{20, w} =$ about 20 S, although considerable hyperimmunization was produced to the three different kinds of antigens. It is important to reemphasize earlier statements that the immune proteins obtained from milk and colostrum or from plasma do not differ in size (2, 3).

The occurrence in all of our bovine and equine samples of a main component of about 7 S with smaller amounts of a component of about 10 S is quite striking and was described earlier in preliminary observations of the bovine proteins (2). It has been suggested that these components (and also the component of 20 S) may simply have a polymeric relationship to one another (2, 14). Thus the protein of 7 S has a molecular weight of about 180,000, and the protein of 10 S would be about double the size. While there has been some suspicion that the component of 10 S may have been produced by the chemical fractionation procedure or by prolonged standing after lyophilization, there is no direct evidence that this is the case. The dried bovine fractions of Cann, Brown, and Kirkwood (10) obtained by a mild physical separation are very similar in sedimentation pattern to those obtained by distinct chemical methods. While lyophilization by itself has not been excluded as leading to the formation of the components of 10 S, we have found that the same preparations investigated after standing more than 3 years in the dry state give precisely the same sedimentation pattern as they did when studied soon after drying.

Recently, Savat³ (19) has attempted to find some method of producing heavier components from bovine or equine globulins which possess sedimentation constants near 7 S. Many of the bovine γ -globulin preparations were molecularly monodisperse. However, she was unsuccessful in altering the amount of heavy material in some samples by allowing the globulin to stand as an ethanol paste, by drying, or by mild heating. But, since freshly prepared samples of γ -globulin obtained by electrophoretic separation contained only the component of 7 S, she assumed that the component of 10 S is somehow produced during the fractionation procedure. The γ -globulins prepared from various species by Nichol and Deutsch (20) also showed only the components of 7 S.

Some evidence has already been adduced which indicates that the components of 10 S are not fortuitous impurities but are truly γ -globulins. This is particularly indicated by the observations that the same components occur in the various equine and bovine fractions whether obtained from plasma or from milk or colostrum. Immunologically, all of the human γ -globulin fractions cross-react, although particles of different size are represented in the preparations (21).

SUMMARY

- 1. Bovine immune proteins obtained by chemical fractionation methods from plasma, milk, or colostrum have been examined in the ultracentrifuge. In each instance, the main component (80 to 90 per cent of the total sedi-
- ³ We are grateful to Dr. H. F. Deutsch for lending us a copy of this thesis and permitting us to cite certain of the results.

menting material) has a sedimentation constant of about 7 Svedberg units, and is always associated with a second component of about 10 Svedberg units.

- 2. The bovine γ -globulins isolated by the electrophoresis-convection method also contain the same two components found in other preparations.
- 3. Equine γ and T-globulins containing high tetanus antitoxic activity likewise contain components which sediment at about 7 S and 10 S; larger particles could not be detected in these preparations.
 - 4. The nature of the components of 10 S is discussed.

BIBLIOGRAPHY

- 1. Smith, E. L., J. Biol. Chem., 164, 345 (1946):
- 2. Smith, E. L., J. Biol. Chem., 165, 665 (1946).
- 3. Smith, E. L., J. Dairy Sc., 31, 127 (1948).
- 4. Smith, E. L., and Gerlough, T. D., J. Biol. Chem., 167, 679 (1947).
- Smith, E. L., Brown, D. M., Fishman, J. B., and Wilhelmi, A. E., J. Biol. Chem., 177, 305 (1949).
- 6. Smith, E. L., and Holm, A., J. Biol. Chem., 175, 349 (1948).
- 7. Hansen, R. G., Potter, R. L., and Phillips, P. H., J. Biol. Chem., 171, 229 (1947).
- 8. Hess, E. L., and Deutsch, H. F., J. Am. Chem. Soc., 70, 84 (1948).
- 9. Hess, E. L., and Deutsch, H. F., J. Am. Chem. Soc., 71, 1376 (1949).
- Cann, J. R., Brown, R. A., and Kirkwood, J. G., J. Am. Chem. Soc., 71, 1609 (1949); 71, 2687 (1949).
- 11. Kabat, E. A., J. Exp. Med., 69, 103 (1939).
- 12. Kabat, E. A., J. Immunol., 47, 513 (1943).
- Williams, J. W., Petermann, M. L., Colovos, G. C., Goodloe, M. B., Oncley, J. L., and Armstrong, S. H., J. Clin. Invest., 23, 433 (1944).
- Oncley, J. L., Scatchard, F., and Brown, A., J. Phys. and Colloid Chem., 51, 184 (1947).
- 15. Deutsch, H. F., and Nichol, J. C., J. Biol. Chem., 176, 797 (1948).
- Pappenheimer, A. M., Jr., Lundgren, H. P., and Williams, J. W., J. Exp. Med., 71, 247 (1940).
- 17. Petermann, M. L., and Pappenheimer, A. M., Jr., J. Phys. Chem., 45, 1 (1941).
- 18. Rothen, A., J. Gen. Physiol., 25, 487 (1942).
- 19. Savat, R. S., Thesis, University of Wisconsin (1949).
- 20. Nichol, J. C., and Deutsch, H. F., J. Am. Chem. Soc., 70, 80 (1948).
- Jager, B. V., Smith, E. L., Nickerson, M., and Brown, D. M., J. Biol. Chem., 176, 1177 (1948).

ON THE RENEWAL OF THE PURINES OF THE DESOXY-PENTOSE AND PENTOSE NUCLEIC ACIDS*

By SIDNEY S. FURST,† PAUL M. ROLL, AND GEORGE BOSWORTH BROWN

(From the Laboratories of the Sloan-Kettering Institute for Cancer Research,

New York)

(Received for publication, April 20, 1949)

The nucleic acids fall into two broad categories. The desoxypentose nucleic acids (DNA), in mammalian tissues, are found only in cell nuclei associated with the chromosomes (1, 2). The pentose nucleic acids (PNA) are chiefly cytoplasmic (3), but are also found in smaller amounts in the nucleolus (4) and in the residual chromosomes (5, 1). Various studies have shown that the phosphorus of the desoxypentose nucleic acids is renewed at a slower rate than that of the pentose nucleic acids.

The data presented here indicate that the rate of incorporation of isotopically labeled adenine into the purines of the DNA is extremely slow and, in fact, is not incompatible with the rate of cell mitosis. On the other hand, the purines of the PNA are shown to be in a state of rapid dynamic equilibrium.

Results

The initial observation that labeled dietary adenine was incorporated into nucleic acids, and that it could also serve as a precursor of nucleic acid guanine (6) was made on nucleic acid preparations which were mixtures of the two types of nucleic acids.

A preliminary experiment was then performed (7) in which the PNA and DNA, from the viscera of rats fed isotopically labeled adenine, were fractionated. Adenine and guanine were then isolated from each of the nucleic acid fractions. Isotope analyses (Table III) of each of the purines so obtained revealed that 15.9 per cent and 9.1 per cent respectively of the adenine and guanine of the PNA were derived from the fed material, while only 0.55 per cent and 0.38 per cent respectively of the adenine and guanine of the DNA were derived from the same material. These figures indicate a renewal of DNA purines which is only 3.5 per cent of that of PNA purines.

To determine the extent to which the small amount of isotope found in

^{*} The authors wish to acknowledge the assistance of the National Cancer Institute of the United States Public Health Service, the Office of Naval Research, and James Foundation of New York, Inc.

[†] Present address, Yale University School of Medicine, New Haven, Connecticut.

the DNA fraction could have been contributed by contaminating PNA, an experiment was performed in which the effectiveness of the method used to separate the nucleic acids (a modification of the Schmidt-Thannhauser procedure (8, 9)) was tested by means of an isotope dilution technique. A sample of isotopically labeled yeast nucleic acid (10) was added to a sample of rat liver and the mixture was fractionated. The DNA fraction first obtained was found to contain 0.092 atom per cent excess N¹⁶, as compared with 2.11 atom per cent excess N15 found in the PNA fraction (Table I). These figures indicate that the DNA fraction contained 4.4 per cent PNA as a contaminant. With the expectation of obtaining further purification, the precipitated DNA was redissolved in alkali and was again precipitated by acid. The twice precipitated material so obtained was found to contain only 0.021 atom per cent excess N15, which in turn corresponds to 1 per cent of the isotope concentration present in the PNA fraction. Thus, from the isotope analyses of the first and second DNA fractions it was seen (Table I) that reprecipitation1 reduced the contamination of the DNA with PNA by a factor of 4. In all succeeding separations of the two types of nucleic acids the DNA was therefore reprecipitated before any further breakdown of this fraction was attempted. In view of the results of this experiment it is apparent that in the experiment on rat viscera, in which a single precipitation of the DNA was employed. a large part of the isotope found in the DNA fraction is to be attributed to the presence of contaminating PNA.

To determine whether the failure of dietary adenine to be incorporated to any extent into DNA was due to the slow renewal of the DNA in resting tissues, or whether, as recently suggested by Reichard (12), this observed failure was due to the fact that adenine cannot be utilized for the synthesis of the purines in DNA, the metabolism of dietary adenine in non-growing and in rapidly regenerating rat livers was studied.

A preliminary experiment showed that, after the feeding of adenine for a single day, the uptake of isotopic nitrogen into the total nucleic acids of different organs varied markedly. It was greatest in the liver (Table II). Isotopically labeled adenine was therefore fed to ten normal adult rats, and PNA and DNA were isolated from the livers alone. After the DNA fraction had been reprecipitated from alkali, total purines, and then adenine, were isolated from it, while adenine and guanine were isolated from the PNA. In non-growing liver, isotope analyses showed DNA and PNA adenine to be derived from dietary adenine to the extent of 0.29 and 21.2 per cent respectively (Table III). If it is assumed that the DNA is un-

¹ Recently, von Euler, Hevesy, and Solodkowska (11) have used the Schmidt and Thannhauser method with a reprecipitation from alkali. Some decrease in the P²⁸ content of the reprecipitated DNA fraction was found there.

TABLE I
Fractionation of Liver Nucleic Acids with Added Labeled Yeast Nucleic Acid
(Experiment III)

Nucleic Acid Analyses

	DNA	PNA
	 mg.	mg.
Estimated in liver sample	33	150
Yeast nucleic acid added	 1.4	305
Total hydrolysate	 27	432
PNA fraction	 Nil	431
Supernatant from 2nd DNA pptn		5
DNA fraction, twice pptd.	 3 7	Nil

Isotope Analyses*

	Atom per cent excess N ¹⁸	Calculated on basis of 100 per cent in purines of PNA
Purines from PNA fraction.	2.11	100
DNA, 1st pptn.	0.092	4.4
" 2nd " .	0.021	1.0
Copper purines from twice pptd. DNA	0.027	1.3

^{*} Determinations made on Consolidated-Nier mass spectrometer model 21-201; probable error ± 0.001 per cent.

Table II

Incorporation of Adenine into Mixed Nucleic Acids of Various Organs (Experiment II)

	Atom per cent excess N ¹⁸	Calculated on basis of 100 per cent in adenine fed
Dietary adenine	6,30	100
Liver, total purines	0 424	6.7
" adenine	0.549	8.7
" guanine	0.229	3.7
" silver pyrimidines	0.019	0.3
Kidney, total purines	0.301	4.8
Intestine, total purines	0.151	2.4
" adenine	0.285	4.5
" guanine	0.089	1.4
Spleen, total purines	0.105	1.7
Testes, " "	0.003	0.0
Urinary allantoin	1.058	16.8

TABLE III
Incorporation of Adenine into Nucleic Acids

			ï	DNA					PNA	V,		
	Total 1	Total purines	Adeninc	nine	Guanine	nine	Total p	Total purines	Adeı	Adenine	Gua	Guanine
Experiment No.	Atom per on cent excess	lculated basis of 100 per cent in idenine fed	Atom per cent excess cent in Nis adenine fed	alculated basis of 100 per cent in adenine fed	Atom per ent excess Nis	alculate n basis 100 per cent in adenine fed	Atom per cent excess N15	Calculated Of Atom per 100 per cent excess cent in adenine fed	Atom per on basis of cent excess cent in adenine fed	lculated basis of 100 per cent in adenine fed	Atom per int excess Nu	Calculated on basis of 100 per cent in adenine fed
I. Mixed viscera, aden-			0.035	0.55	0.024	0.38			1.00	15.9	0.57	9.1
IV. Non-growing liver,	0.008	0.17	0.014	0.3		Millionia y Alexandro and Alex			1.02	21.2	0.415	8.7
V. Regenerating liver,	0.419	8.7	0.78	16.3	0.15	3.2	0.566	11.8	1.09	22.7	0.388	8.1
VI. Regenerating liver, 26 days, no supple-	0.308	6.5					0.087	8:	0.132	2.7	0.080	1.7
ment after 5 days												

contaminated by PNA, the renewal of DNA adenine is 1.37 per cent of that of the PNA adenine, or an uncorrected ratio of the PNA to the DNA of 73:1; and if only a quarter of the isotope value of the DNA fraction is attributed to contaminating PNA, the ratio² of the renewals of liver PNA purines to DNA purines becomes 100:1.

The same procedures were employed to study the uptake of dietary adenine in the liver nucleic acids of rats during the first 5 days following subtotal hepatectomy. Although the incorporation of dietary adenine into the purines of the PNA of the growing liver (22.7 per cent) was approximately the same as that in non-growing liver, the incorporation into the DNA fraction was enormously increased, and amounted to 16.3 per cent, or 75 per cent of the value found for the purines of the PNA fraction (Table III). This provides evidence that dietary adenine does function as a precursor of the DNA purines when mitosis is occurring and testifies that the small amount of N¹⁵ incorporated into the DNA purines of non-growing liver must be indicative of an almost negligible renewal of the DNA purines in non-growing tissues.

Additional confirmation of the extremely slow renewal of the DNA purines came from another experiment in which three partially hepatectomized rats were fed isotopic adenine for 5 days and were then continued without further supplement of adenine for 21 days. At the end of this period the isotope value (0.309) of the total DNA purines obtained from the liver was not much lower than the 5 day value (0.419) of the previous experiment. In contrast, the isotope values of the PNA purines had fallen markedly, indicating that they had been extensively replaced by nonisotopic precursors during this period (Table III). In accord with available data on the growth of regenerating rat liver (15-17), the livers removed on the 6th day amounted to 85 per cent of the estimated original liver weight, and those removed on the 27th day amounted to 108 per cent of the original weight. If allowance is made for this additional growth utilizing non-isotopic precursors between the 6th and the 27th days. nearly all of the decrease in the isotope value of the DNA purines is accounted for.

EXPERIMENTAL

Feeding and Maintenance of Experimental Animals—The rats used in all experiments were adult, male, Sherman strain animals averaging 300 gm. in weight. They were housed in individual metabolism cages and were

² Nitrogen from biological sources contains about 0.005 atom per cent more N¹⁵ than does commercial tank nitrogen (13, 14) and the values are all reported here as atom per cent excess over tank nitrogen. The subtraction of 0.005 from each value would result in an appreciable increase in the ratios.

kept on a diet of pulverized, moistened Purina chow pellets. The adenine, labeled in the 1 and 3 positions with isotopic nitrogen (6, 18) was admixed with this diet, and the daily allotment of food was adjusted so that each animal received 0.2 mm of adenine (except in Experiment II; 0.4 mm) and 50 gm. of chow (dry weight) per kilo of body weight per day. On the afternoon of the day following the last feeding the animals were sacrificed either by decapitation or by injection of urethane followed by injection of magnesium sulfate. The organ or organs to be worked up were quickly removed and frozen on dry ice. They were stored at -30° until further treatment.

Subtotal Hepatectomy Technique (15)—Adult rats, without any previous treatment, were anesthetized with ether and the left lateral and both central lobes of the liver were resected $\frac{1}{4}$ inch distal to the ligature. A small amount of sulfadiazine was sprinkled on the wound. Feeding was commenced on the day following the operation.

Fractionation of DNA and PNA from Tissues—A modification of the Schmidt-Thannhauser procedure (8) was used in all experiments as follows: The weighed, frozen tissues were homogenized with 10 volumes of water in a Waring blendor at 5°, and an equal volume of cold, 14 per cent trichloroacetic acid was added to the homogenate. The resulting mixture was stirred for half an hour, and was then centrifuged in 250 cc. centrifuge tubes. The residue was washed by centrifugation, once with 1 per cent trichloroacetic acid, then with water, with anhydrous ethanol, and finally with ether. All operations were carried out at 5°. The tissue powder was then defatted by hot extraction for 2 hours with 1:1 methanol and chloroform. The desiccated tissue was placed in a rubber-stoppered Erlenmeyer flask with 1 N KOH (10 cc. per gm. of fresh tissue), and incubated at 37° for 20 hours, after which time all the tissue was in solution. After small aliquots were removed for DNA (19) and PNA (20, 21) analyses, the DNA fraction was precipitated from the total hydrolysate by addition, with rapid stirring and ice cooling, of one-tenth the volume of 25 per cent trichloroacetic acid, and enough 6 N HCl to make the solution 0.1 N with respect to HCl. When precipitation was complete, most of the supernatant was removed through a filter stick, and the precipitated DNA fraction was finally collected by centrifugation. The supernatant (PNA) was filtered through Celite to eliminate last traces of suspended solids, and an aliquot was removed for analysis. The solids were washed once with 2.5 per cent trichloroacetic acid, centrifuged, and the supernatant discarded. In all but the rat viscera experiment, the DNA fraction was redissolved in 1 N KOH (1 cc. per gm. of fresh tissue) by stirring at room temperature for 20 minutes, and then reprecipitated with proportionate amounts of trichloroacetic and hydrochloric acids. The DNA was then extracted

from the total residue with 5 per cent trichloroacetic acid (0.5 cc. per mg. of DNA estimated to be present) by heating with stirring at 90° for 20 minutes. The extract was separated by centrifugation, and the residue was then reextracted with one-half the previous volume of 5 per cent trichloroacetic acid. The extracts were combined, filtered to eliminate last traces of solids, and an aliquot was removed for analysis.

Isolation of Purines from DNA and PNA Fractions—Purines were isolated from the two nucleic acid fractions as either the copper or the silver salts.

To isolate purines in the form of their copper salts from a PNA fraction, the filtered supernatant was used as such; to isolate copper purines from DNA, the hot trichloroacetic acid solution of the DNA was used. In either case 10 N H₂SO₄ was added to make the solution 0.4 N with respect to H₂SO₄, and hydrolysis was accomplished by refluxing for 2 hours. was cooled, adjusted to pH 9 with NaOH, and then to pH 5 with dilute H₂SO₄. It was then reheated in a water bath, and the copper purines were precipitated with NaHSO₃ and CuSO₄ according to Hitchings (22). The solution was vigorously stirred throughout the course of the addition of reagents. The supernatant was largely removed through a filter stick. and the precipitated copper purines were collected and washed twice with hot water by centrifugation. The washed material was dissolved in a minimal amount of hot 3 N HCl, copper was removed by precipitation with H₂S, and the CuS was washed with hot dilute HCl. Last traces of H₂S were removed from the combined filtrate and washings by aeration. Guanine was then allowed to precipitate at about pH 6, and adenine was isolated as the picrate from the supernatant (23). The guanine was crystallized as the sulfate from 1 N H₂SO₄ and recrystallized one or more times. The adenine picrate was twice recrystallized from 25 per cent acetic acid. A portion of the adenine picrate was suspended in 0.1 N HCl and, after continuous extraction with ether, the solution of adenine hydrochloride so obtained was used for isotope analysis.

The same PNA and DNA starting materials were used for the isolation of silver purines as were used for isolation of the copper salts. Enough 6 N HCl was added to the solution of the fraction to make it 1 N in HCl, and it was refluxed for 1 hour. The silver purines were precipitated with an excess of ammoniacal silver chloride (24). They were collected by decantation, washed several times with water, and converted to the purine hydrochlorides by treatment with 25 and 10 cc. portions of 3 N HCl (25). The purine hydrochloride solution was filtered through Celite, and guanine and adenine were separated and purified. All final products were analyzed for nitrogen content by the micro-Kjeldahl method.

Preparative Separation of Adenine and Guanine by Counter-Current Dis-

tribution—The purines were recovered from 15 to 30 mg. samples of copper purines by two treatments with 1 N HCl and H₂S in a micro hydrogenation apparatus. The purine hydrochloride solution was concentrated to dryness and dissolved in a few drops of 2 N NaOH and 8 cc. of buffer.

Adequate separation of 5 to 10 mg. quantities of mixtures of adenine and guanine was accomplished in a system of 0.1 m potassium phosphate, pH 9.5, and n-butanol-isopropanol (60:40). In this system the spread between the distribution constants (guanine 0.5, adenine 1.3) is not as great as in the system at pH 6.5 (26), but several times as much guanine may be dissolved in the alkaline system.

Thirty-two transfers, without withdrawals, were made in a twenty-five tube counter-current distribution apparatus (27), at which point the leading edge of the adenine band has just begun to overlap the guanine band. With 5 mg. quantities of adenine in Tube 0 a symmetrical curve is obtained. 5 mg. of guanine yield an unsymmetrical curve, the leading side of which gives evidence of a K corresponding to that obtained with dilute solutions of guanine, but the K for the trailing side is lower than expected, due to inadequate solubility in the upper phase which prevents attainment of an equilibrium between the two phases until after several extractions of Tube 0 have been made. From the patterns obtained from mixtures the leading half of the adenine band and the trailing half of the guanine band were selected. To avoid dilution by extraneous nitrogen it was necessary to concentrate the contents of the selected tubes to about one-quarter volume and to precipitate the copper adenine or copper guanine.

Control experiments with mixtures of isotopic guanine and non-isotopic adenine, and vice versa, showed cross-contaminations of 0.0 to 0.3 per cent of guanine in the adenine fractions selected and 1 to 2 per cent of adenine in the guanine fractions.

In the preparation of the PNA purines from Experiment VI the CuS supernatant, as introduced into Tube 0, contained 0.086 per cent N¹⁵ (copper purines 0.087); the reasonably pure guanine from Tubes 4 through 8 contained 0.080; the mixture from Tubes 10 through 16 contained 0.110; and the pure adenine from Tubes 17 through 24 contained 0.132. In addition the contents of Tubes 0 through 3, which contained a trace of adenine and considerable guanine, were precipitated and analyzed and were found to contain only 0.040 per cent N¹⁵, which indicates the presence of non-isotopic nitrogen in a form possessing a low distribution constant in this solvent pair, possibly cysteine or glutathione. The contamination of copper purine preparations with non-purine nitrogen is not unexpected (22) when these precipitates are prepared directly from an alkaline digest of tissue, as was the case with the copper purines from the PNA fractions. The possible presence of contaminants not absorbing at 260 m μ and hav-

ing distribution constants similar to either of the purines must also be recognized, and the isotope values determined directly on the copper purine preparations must be considered as minimal values.

Experiment I. Incorporation of Dietary Adenine into DNA and PNA of Pooled Rat Viscera—Five rats totaling 1510 gm. in weight were used. They were fed adenine containing 6.3 atom per cent excess N¹⁵ (6) for 10 days. The thymus, lungs, heart, spleen, kidneys, liver, stomach, testes, and large and small intestine of each animal were pooled and utilized. A small sample of the defatted tissue was removed for PNA and DNA analyses. The DNA fraction was precipitated only once. Total purines were isolated as the copper salts. All final products were characterized by counter-current distribution (26). In this experiment an inadequate quantity of guanine was obtained from the DNA fraction and it was characterized only by counter-current distribution. Isotope values of the isolated products are given in Table III.

· Analysis—Total mg. of nucleic acid in the following: desiccated tissue, DNA 560, PNA 749; total hydrolysate, DNA 314, PNA 836; DNA fraction, DNA 400, PNA nil; PNA fraction, DNA nil, PNA 781.

DNA fraction; adenine picrate, $C_6H_4N_5 \cdot C_6H_2O_7N_3 \cdot H_2O$, calculated, N 29.3; found, N 28.9.

PNA fraction; adenine picrate, calculated, N 29.3; found, N 28.9; guanine, $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot H_2O$ (dried at 130°), calculated, N 33.4; found, N 34.2; $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$ (rehydrated and dried at 20°), calculated, N 32.1; found, N 32.3.

Experiment II. Incorporation of Adenine into Total Nucleic Acids of Various Rat Organs—Eight rats, weighing a total of 2516 gm., were fed adenine (6.30 atom per cent excess N¹⁵) at a level of 0.4 mm (54 mg.) per kilo of body weight per day for a single day. The pooled livers, kidneys, spleens, testes, and intestines were individually worked up for total nucleic acids (6). In the cases of the livers and intestines sufficient nucleic acids were available to permit the separation of adenine and guanine, but from the other organs copper purines only were prepared. Allantoin was isolated from the pooled urines. The isotope concentrations of all final products are given in Table II.

Experiment III. Fractionation of Liver Nucleic Acids with Added Labeled Yeast Nucleic Acid—17.2 gm. of normal rat liver, estimated to contain 150 mg. of PNA and 33 mg. of DNA, were worked up as usual but with the addition of 305 mg. of purified yeast nucleic acid (containing 3.35 atom per cent excess N¹⁵) (10) just before hydrolysis in 1 N KOH. The solution containing once precipitated DNA, as well as the trichloroacetic

³ Low values were always obtained on this fraction, presumably due to interference by other constituents of the hydrolysate.

acid extract containing twice precipitated DNA, was subjected to isotope analysis. Copper purines were isolated from the PNA fraction and from the twice precipitated DNA fraction. The results of nucleic acid analyses and of isotope determinations on all final and intermediate products are indicated in Table 1.

Experiment IV. Incorporation of Adenine into PNA and DNA of Non-Growing Rat Liver—Ten rats, totaling 3200 gm., were fed adenine (4.80 atom per cent excess N¹5) (18) for 5 days. A total of 87 gm. of liver tissue, representing 2.7 per cent of the total body weight, was removed at autopsy. The pooled livers were worked up as described above. All purines were isolated as the silver salts. Insufficient guanine was obtained from the DNA fraction to be able to prepare an adequately purified sample. Results of isotope determinations are indicated in Table III.

Analysis—Total mg. of nucleic acid in the following: desiccated liver, DNA 140, PNA 768; once precipitated DNA, DNA 117, PNA 38; twice precipitated DNA, DNA 165, PNA nil; PNA fraction, DNA nil, PNA 760.

DNA fraction; adenine picrate, C₆H₅N₅·C₆H₂O₇N₂·H₂O, calculated, N 29.3; found, N 29.5.

PNA fraction; adenine picrate, calculated, N 29.3; found, N 30.2; guanine, $(C_bH_bON_b)_2 \cdot H_2SO_4 \cdot 2H_2O$, calculated, N 32.1; found, N 32.5.

Experiment V. Incorporation of Dietary Adenine into PNA and DNA of Regenerating Rat Liver—Seventeen rats were partially hepatectomized. Starting on the day following the operation, and continuing for 5 days thereafter they were fed adenine (containing 4.80 atom per cent excess N¹⁵), and were sacrificed on the day following the last feeding. In the strain and weight class of rats used in these experiments we have found that the intact liver averages 2.7 per cent of the total body weight. The total weight of all rats employed in this experiment was 4880 gm. and the total weight of their intact livers was therefore estimated to be approximately 133 gm. Of this, 89.9 gm., or 67 per cent (range 54 to 80), were removed at operation. A total of 113.7 gm., or 85 per cent (range 71 to 117) of the estimated original weight, was obtained at autopsy. This leads to an estimate that, of the liver tissue used in this experiment, approximately 62 per cent by weight was newly formed during the 6 days following the operation.

The regenerated livers obtained at autopsy were treated as were those obtained in the experiment with normal liver. Part of each nucleic acid fraction obtained was used for the preparation of copper purines, the isotope content of which is reported under total purines. Silver purines were isolated from the remainder of each fraction. Adenine and guanine were

⁴ This unexpectedly high value is possibly due to analytical error.

isolated from the silver purines of the PNA, but an attempt to isolate these compounds from the silver purines of the DNA was unfortunately unsuccessful. In order to obtain DNA adenine and guanine for isotope determinations, a portion of the DNA copper purines was separated by means of the counter-current distribution procedure. Essentially pure adenine was obtained in this way, but the guanine fraction obtained was estimated to be contaminated with adenine to the extent of possibly 10 per cent. The results of isotope determinations on all products are given in Table III.

Analysis—Total mg. of nucleic acid in the following: total hydrolysate, DNA 176, PNA 1312; twice precipitated DNA, DNA 225, PNA nil; PNA fraction, DNA nil, PNA 1321.

PNA fraction; adenine picrate, C₆H₆N₆·C₆H₂O₇N₂·H₂O, calculated, N 29.3; found, 30.0; guanine, (C₆H₆ON₆)₂·H₂SO₄·2H₂O, calculated, N 32.1; found, N 32.7.

Experiment VI. Retention of Isotopic Adenine Once Incorporated into PNA and DNA of Regenerating Rat Livers—Three rats, totaling 836 gm., we're partially hepatectomized, and 18.6 gm. of liver were removed. After being fed adenine (4.80 atom per cent excess N¹⁵) for 5 days, the animals were maintained on an unsupplemented diet for an additional 21 days. They were sacrificed on the 27th day after operation, and 24.7 gm. of liver tissue were obtained at autopsy. This represents 108 per cent (range 103 to 111) of the estimated original weight, and 83 per cent of this weight is estimated to have been formed during the 26 day period. The tissues were worked up as usual, and copper purines were isolated from both the PNA and DNA fractions. Adenine and guanine were separated from the PNA copper purines by counter-current distribution, but since only about 4 mg. of purines were present in the total DNA fraction, no attempt to separate them was warranted. Isotope analyses of final products are given in Table III.

Analysis—Total mg. of nucleic acid in the following: total hydrolysate, DNA 31' PNA 200; twice precipitated DNA, DNA 41, PNA nil; PNA fraction, DNA nil, PNA 180.

DISCUSSION

The several studies of nucleic acid phosphorus metabolism, which have recently been reviewed by Hevesy (28), have shown that the rate of renewal of the phosphorus of the DNA fractions is slower than that of the PNA fractions. However, none of the work with P³² has shown so marked a difference between the rates of replacement of the PNA and the DNA phosphorus as has been observed here for the PNA and DNA purines. Hammarsten and Hevesy (29) found that for the total rat the ratio of the turnovers of PNA phosphorus to DNA phosphorus was 1.66:1 after 2

hours, and for individual organs was 33:1 in the liver, 2:1 in the intestine, and 3:1 in the spleen. The ratio of PNA phosphorus to DNA phosphorus of 33:1 for liver is the only one even approaching the uncorrected ratio of 73:1 which we have observed for the relative incorporation of adenine into the PNA and DNA purines. None of the phosphorus figures on total viscera are of a comparable magnitude.

The differences between the relative renewals observed with adenine and with phosphate might be explained by the replacement of a small amount of the DNA phosphorus when no change was occurring in the carbon-nitrogen skeleton of the molecule.

Marshak (30) has recently demonstrated that, after the administration of P³², no significant amount of P³² is liberated by the action of desoxyribonuclease from nuclei isolated from non-growing tissue, while much P³² is liberated by the action of ribonuclease. The histological localization of newly incorporated P³² in the DNA of tissues of high mitotic rate has recently been demonstrated by radioautographic methods (31).

Brues, Tracy, and Cohn (32), using a different method for the separation of the two types of nucleic acids, found that in rat liver the ratio of turnovers of PNA phosphorus to DNA phosphorus was 5.2:1 on the 3rd day after and 5.9:1 on the 8th day after a single injection of P³². Brues has commented (33) that in subsequent experiments higher ratios have been obtained. These authors found that in regenerating liver there was a proportionately greater incorporation into, and a longer retention of, the phosphate of the DNA fraction.

When isotopic ammonia was fed (34), a negligible uptake was obtained in the desoxypentose nucleic acids, but the isotope values encountered were so low that a reliable ratio of the rates of renewal could not be calculated. Hammarsten and coworkers (35) have shown that the nitrogen of N¹⁵-labeled glycine is incorporated into the DNA purines of rat livers at a slower rate than into the PNA purines and that the PNA fraction associated with isolated nuclei is renewed more rapidly than is the cytoplasmic PNA fraction. It was also shown that in regenerating liver the purines of the DNA fraction show an increased uptake of the nitrogen of the glycine, a picture generally comparable to the one found with adenine. However, in the experiments with glycine, the renewal of purine nitrogen in the DNA fraction of non-growing liver was considerable, and the ratio of the rates of renewal of the purine nitrogen of the total liver PNA fraction and the DNA fraction was 4:1, a ratio of an entirely different magnitude from that found with adenine.

It was also found (35) that with glycine the incorporation into the purines of the PNA of regenerating liver was much greater than into the PNA purines of non-growing liver. On the other hand, in these experi-

ments with adenine, the incorporation into the PNA purines of non-growing or of regenerating liver was essentially identical. Rittenberg, Sproul, and Shemin (36) have found that the incorporation of glycine into the proteins of non-growing or into regenerating liver was at about the same rate and they concluded that growth was the result of the inhibition of degradative reactions rather than the increase of the synthetic processes. The same interpretation could be applied to the results obtained here for adenine incorporation into the PNA.

In the data reported here the relative introduction of N¹⁵ of the dietary adenine into the nucleic acid guanine and the nucleic acid adenine has not been as constant as it appeared at first (37). In the original experiments involving the total nucleic acids of mixed viscera, the relative incorporation of the N¹⁵ into the two purines was in the ratio of 0.60 and 0.59 (6), while in the present experiment with mixed viscera it was 0.57 in the purines of the PNA fraction. However, in the total nucleic acids of individual organs the ratio of the isotope in the guanine to that in the adenine was 0.31 for intestine and 0.42 for liver. In the separated PNA fractions, it was 0.41 for the non-growing liver and 0.36 for the regenerating liver.

An estimate may be made of the biological half life of the purines of the PNA fraction from the decrease in the isotope concentration between the 6th and 27th days (Experiments V and VI). The isotope value for the copper purines fell in 21 days from 0.566 to 0.087, or to 15 per cent of its initial value. If the loss of N¹⁵ follows a first order course, the half life computed from these values is nearly 8 days, while that computed from the corresponding adenine values is 7 days, and from the guanine values is 9 days. If allowance is made for the growth occurring between the 6th and 27th days, a somewhat longer half life is indicated. It is interesting that this biological half life of about 8 days for the purine ring nitrogen of the combined pentose nucleic acids of liver in the latter stages of regeneration is of approximately the same order as that of the protein nitrogen of non-growing liver, which was shown by Shemin and Rittenberg (38) to be 7 days. This dynamic state of the pentose nucleic acids may well be correlated with a functional rôle in the metabolic processes of the cell.

The lowest values for the absolute rate of renewal of the phosphorus of DNA, calculated from the P²² value of the inorganic phosphate, have indicated that a replacement of about 0.1 per cent of the DNA phosphorus of the liver or kidneys occurs in a 2 hour period (29), or a rate of 1 per cent per day (39), a rate which could not be accounted for solely by the new DNA arising through mitosis (28). In the case of nucleic acid adenine, it is not possible to determine the concentration of the isotope in the direct precursor, since the relative contributions of the labeled exogenous adenine and non-labeled material elaborated in vivo are unknown. However,

from the estimated half life of the PNA purines of about 8 days, it may be calculated that 8.5 per cent renewal would occur per day, or about 36 per cent in 5 days. From this value and the observed ratio of the rates of renewal of the PNA and DNA purines it may be estimated that, if the proportions of isotopic and non-isotopic precursors of the two types of nucleic acids are the same, the maximum renewal of the DNA purines in non-growing tissue is of the order of 0.4 per cent in the 5 day period. This is appreciably less than the renewal rate for the phosphorus, and a value not incompatible with available estimates of the rate of mitosis occurring in non-growing liver (17, 28, 35, 40).

Thus, it is conceivable that all of the isotope incorporated into the DNA purines was introduced in the process of formation of new nuclei, and that, once it is formed, the skeleton of the DNA is not at all in dynamic equilibrium with other constituents of the cell. This can be correlated with the cytological observation of Ris (41) that the chromosomal components double in the course of mitosis, and the recent report by Vendrely and Vendrely (42) that, within a species, chemical analyses indicate a constant DNA content per nucleus and that the amount of DNA in haploid nuclei is one-half that in diploid nuclei. Inhibitors of desoxyribonuclease, such as those recently reported (43, 44), may function to maintain this stability of the DNA throughout the interphase period of the cell.

The significance of the fact that the desoxypentose nucleic acids are an exception to the general concept of the rapid dynamic equilibrium of body constituents (45) is not obvious, but this biochemical stability furnishes a desirable (46) permanence which adds to the circumstantial evidence that desoxyribonucleic acid may be a fundamental component of the gene.

The authors wish to express appreciation for the advice of Dr. Mary L. Petermann, the assistance of Helen Getler, the aid of Audrey Larack and Catherine Lamb in performing the nucleic acid analyses, the aid of John Deonarine with the isotope analyses, the cooperation of Dr. Harold Beyer with the mass spectrometer, and to Roscoe C. Funk, Jr., for the microanalyses.

SUMMARY

In both total viscera and in non-growing liver of rats it has been shown that isotopically labeled dietary adenine is rapidly incorporated into the purines of the pentose nucleic acid fraction, but that the incorporation into the purines of the desoxypentose (nuclear) nucleic acid fraction is about 1 per cent as extensive.

During the period of rapid regeneration of the liver of partially hepatectomized rats it was found that the incorporation of purines into the des-

oxypentose nucleic acids was about 75 per cent of that into the pentose nucleic acid fraction. When similarly treated animals were then allowed an unsupplemented diet for 3 weeks, the purines of the pentose nucleic acids were largely replaced by non-isotopic precursors, while the purines of the desoxypentose nucleic acids were replaced to but a small extent.

An isotope dilution analysis of the method used for the separation of the two classes of nucleic acids has been carried out.

A survey of the incorporation of dietary adenine into several organs of the rat is reported.

The extent of incorporation of dietary adenine into the desoxypentose nucleic acids of liver can be roughly correlated with the extent of cell mitosis, while the dynamic state of the purines of the pentose nucleic acids of liver is comparable to that of the protein of liver.

BIBLIOGRAPHY

- 1. Mirsky, A. E., and Ris, H., J. Gen. Physiol., 31, 7 (1947).
- 2. Caspersson, T., Skand. Arch. Physiol., suppl. 8, 73 (1936).
- 3. Caspersson, T., and Schultz, J., Nature, 142, 294 (1938).
- Caspersson, T., and Santesson, L., Studies on protein metabolism in cells of epithelial tumors, Stockholm (1942).
- 5. Brachet, J., Embryologie chimique, Paris (1944).
- Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., J. Biol. Chem., 172, 469 (1948).
- Brown, G. B., Petermann, M. L., and Furst, S. S., J. Biol. Chem., 174, 1043
 (1948).
- 8. Schmidt, G., and Thannhauser, S. J., J. Biol. Chem., 161, 83 (1945).
- 9. Schneider, W. C., J. Biol. Chem., 164, 747 (1946).
- Di Carlo, F. J., Schultz, A. S., Roll, P. M., and Brown, G. B., J. Biol. Chem., 180, 329 (1949).
- 11. von Euler, H., Hevesy, G., and Solodkowska, W., Ark. Kemi, Mineral. o. Geol., 26 A, 1 (1948).
- 12. Reichard, P., J. Biol. Chem., 179, 773 (1949).
- 13. Schoenheimer, R., and Rittenberg, D., J. Biol. Chem., 127, 285 (1939).
- Geren, W., Bendich, A., Bodansky, O., and Brown, G. B., J. Biol. Chem., 183, 21 (1950).
- 15. Higgins, G. M., and Anderson, R. M., Arch. Path., 12, 186 (1931).
- 16. Brues, A. M., Drury, D. R., and Brues, M. C., Arch. Path., 22, 658 (1936).
- 17. Stowell, R. E., Arch. Path., 46, 164 (1948).
- 18. Cavalieri, L. F., Tinker, J. F., and Bendich, A., J. Am. Chem. Soc., 71, 533 (1949).
- 19. Dische, Z., Mikrochemie, 8, 4 (1930).
- 20. Schneider, W. C., J. Biol. Chem., 161, 293 (1945).
- 21. Albaum, H. G., and Umbreit, W. W., J. Biol. Chem., 167, 369 (1947).
- 22. Hitchings, G. H., and Fiske, C. H., J. Biol. Chem., 140, 491 (1941).
- 23. Levene, P. A., J. Biol. Chem., 53, 441 (1922).
- 24. Salkowski, E., Arch. ges. Physiol., 69, 280 (1898).
- 25. Kerr, S. E., and Seraidarian, K., J. Biol. Chem., 159, 211 (1945).
- 26. Tinker, J. F., and Brown, G. B., J. Biol. Chem., 173, 585 (1948).

- 27. Craig, L. C., J. Biol. Chem., 155, 519 (1944).
- 28. Hevesy, G., Radioactive indicators, New York, 325 (1948).
- 29. Hammarsten, E., and Hevesy, G., Acta physiol. Scand., 11, 355 (1946).
- 30. Marshak, A., J. Cell. and Comp. Physiol., 32, 381 (1948).
- 31. Leblond, C. P., Stevens, C. E., and Bogoroch, R., Science, 108, 531 (1948).
- 32. Brues, A. M., Tracy, M. M., and Cohn, W. E., J. Biol. Chem., 155, 619 (1944).
- 33. Brues, A. M., Cold Spring Harbor Symposia Quant. Biol., 12, 222 (1947).
- 34. Davidson, J. N., and Raymond, W., Biochem. J., 42, p. xiv (1948).
- Berstrand, A., Eliasson, N. A., Hammarsten, E., Norberg, B., Reichard P., and von Ubisch, H., Cold Spring Harbor Symposia Quant. Biol., 13, 22 (1948).
- 36. Rittenberg, D., Sproul, E. E., and Shemin, D., Federation Proc., 7, 180 (1948).
- 37. Brown, G. B., Cold Spring Harbor Symposia Quant. Biol., 13, 43 (1948).
- 38. Shemin, D., and Rittenberg, D., J. Biol. Chem., 153, 401 (1944).
- 39. Hevesy, G., and Ottesen, J., Acta physiol. Scand., 5, 237 (1943).
- 40. Brues, A., and Marble, B., J. Exp. Med., 65, 15 (1937).
- 41. Ris, H., Cold Spring Harbor Symposia Quant. Biol., 12, 158 (1947).
- 42. Vendrely, R., and Vendrely, C., Experientia, 4, 434 (1948).
- 43. Zamenhof, S., and Chargaff, E., Science, 108, 628 (1948).
- 44. Dabrowska, W., Cooper, E. J., and Laskowski, M., J. Biol. Chem., 177, 991 (1949).
- 45. Schoenheimer, R., The dynamic state of body constituents, Cambridge (1946).
- 46. Schrödinger, E., What is life?, London (1948).

THE DIRECT OXIDATION OF ADENINE IN VIVO*

By AARON BENDICH, GEORGE BOSWORTH BROWN, FREDERICK S. PHILIPS, AND J. B. THIERSCH†

(From the Protein Section and the Pharmacology Section of the Sloan-Kettering Institute for Cancer Research, New York)

(Received for publication, October 19, 1949)

When large quantities of adenine are administered to various mammalian species, extensive crystalline masses, which for some time were mistaken to be uric acid (1), deposit in the kidneys (1-3). Nicolaier (2) believed the isolated transformation product to be 2,8-dioxyadenine (4), and this was confirmed by Fischer (2), although the experimental data leading to the elucidation of the structure were never reported. Interest in this problem was renewed 48 years after the initial observation by the report that large amounts of adenine produce a rise in blood pressure and a syndrome resembling avitaminosis (blacktongue) in dogs (5) and a slight increase in the rate of growth of sarcoma 180 in mice (6).

^{*} The authors gratefully acknowledge the assistance of the National Cancer Institute of the United States Public Health Service, the Office of Naval Research, and the James Foundation of New York, Inc.

[†] Postdoctoral fellow of the National Cancer Institute.

We have studied the fate of large doses of adenine, administered either orally or intraperitoneally (Table I), and have observed the deposition of these crystals in kidneys. A rigorous comparison of synthetic 2,8-dioxy-adenine with the natural product from the kidneys of rats which had received adenine was made (Table II).

An examination of the structural formula of adenine (I) reveals that the carbon atoms at positions 2 and 8 are both amidine carbon atoms, and it would be difficult to decide a priori what the course of the oxidation to 2,8-dioxyadenine (IV) might be. Since 2-oxyadenine (isoguanine) occurs naturally in butterfly wings (7) and as the 9-D-riboside (crotonoside) in croton beans (8, 9), whereas 8-oxyadenine has not been found in nature, it appeared reasonable that the conversion of I to IV might occur via a prior oxidation in the 2 position.

Accordingly, isoguanine (II) (10) was tested and it was found, indeed, to serve as a precursor of 2,8-dioxyadenine (IV). However, we have found that the administration of 8-oxyadenine (III) (11) also gives rise to clusters of 2,8-dioxyadenine (IV) crystals in rat kidney. Thus, it seems that, so far as these transformations are concerned, extensive oxidation of adenine can proceed in the 2 or 8 position, irrespective of whether the other position is substituted by oxygen. This is reminiscent of the behavior of hypoxanthine, xanthine, and 6,8-dioxypurine towards xanthine oxidase from milk or ox spleen; in all three cases, uric acid was the final product (12).

Of more immediate pertinence is the report by Booth (13) of the oxidation of adenine by xanthine oxidase from whey. It was found that a product responding to the Benedict uric acid test resulted from the action of this enzyme on adenine. However, since no ammonia was evolved and 2 atoms of oxygen were consumed per molecule of adenine oxidized, it appears likely that the product actually obtained was 2,8-dioxyadenine. The enzyme was active against 8-oxyadenine and somewhat less so against isoguanine; in both cases 1 atom of oxygen per molecule was consumed and no ammonia was evolved, and the products gave a positive Benedict uric acid test. The lack of specificity of the Benedict test is well known, and it is probable that 2,8-dioxyadenine was the final product.

In order to determine whether any of the 2,8-dioxyadenine deposited in the kidney had arisen from "endogenous" adenine of the animal in addition to that administered, adenine, containing 0.492 atom per cent

¹ Dr. T. F. Yü and Dr. A. B. Gutman, Columbia Research Service, Goldwater Memorial Hospital, New York, have had the kindness to determine the chromogenic properties of 2,8-dioxyadenine with the Benedict reagent. It was found that 2,8-dioxyadenine gives almost as much color as does uric acid, and that 2,8-dioxyadenine is not acted upon by uricase.

excess N¹⁵ (labeled in the 1 and 3 positions (14)), was given orally to rats at a level of 500 mg. per kilo. It was found that the 2,8-dioxyadenine isolated from the kidneys contained 0.473 atom per cent excess N¹⁵. From this dilution, it may be calculated that about 20 mg. of adenine per kilo of rat tissue were available, during the 17 hours of the experiment, for admixture with the labeled dietary adenine, participating with the latter in 2,8-dioxyadenine formation.

In another experiment, isoguanine, containing 1.51 atom per cent excess N¹⁵ (10), was given to rats by the intraperitoneal route. The 2,8-dioxyadenine, isolated from the kidneys, contained 1.48 atom per cent excess N¹⁵. It is not necessarily valid to argue from these figures that isoguanine is normally present in the tissues of the rat. The dilution observed in this case is less than that obtained when adenine was given, but it does indicate that a small amount of a precursor leading to 2,8-dioxyadenine is normally present in rat tissue. The observed conversion of isoguanine to 2,8-dioxyadenine is over 20 times the conversion to allantoin, which was determined in conjunction with an isotope dilution analysis of the urinary allantoin.

The isotopic isoguanine experiment afforded an opportunity to investigate, in part, the mechanism whereby adenine is converted to guanine in the rat (15). A reasonable scheme (16) involves the oxidation of adenine (I) in the 2 position to give isoguanine (II). The possibility of such an oxidation is indicated by the studies described here. The next step would then be an amination of the 2-hydroxyl to give 2,6-diaminopurine (V), a compound which is known to be utilized for the biosynthesis of nucleic acid guanine (VI) (17). From the data in Table III, it is seen that iso-

guanine is not utilized by the rat for nucleic acid synthesis. Perhaps the rapid oxidation in the 8 position to yield 2,8-dioxyadenine precludes any significant rôle it might otherwise play in nucleic acid synthesis in the rat.

When the extremely limited solubility of 2,8-dioxyadenine² is taken into consideration, it is not surprising to find kidney deposits of this compound following administration of its precursors in large amount. This process results in renal damage which, if sufficiently severe, may cause uremia and consequently secondary changes in other organs. Current studies of adenine intoxication in rats show that an anemia, resembling the anemias observed in advanced renal diseases in man (18), can develop following severe renal damage.³ The origin of the hypertension and a syndrome resembling "avitaminosis" (blacktongue) in dogs (5) might have to be reconsidered in this light. The results of an investigation of the circumstances under which 2,8-dioxyadenine deposits in kidney, causing a condition we have termed "adenine kidney," will be presented elsewhere.³

It is remarkable that no 2,8-dioxyadenine could be demonstrated in the kidney after it was administered, either orally or intraperitoneally. That this failure to deposit in the kidney is not due to lack of absorption from the peritoneal cavity is demonstrated by the complete absence of 2,8-dioxyadenine in the peritoneal fluid 1 day after its injection. Extensive degradation must have occurred, since none could be demonstrated in the tissues and only trace amounts were found in the urine. The administration of isotopically labeled 2,8-dioxyadenine will be necessary to elucidate its fate.

EXPERIMENTAL

"Adenine Kidney"—Macroscopically, kidneys of animals given adenine, isoguanine, or 8-oxyadenine by the oral or intraperitoneal route were enlarged, pale, and, in advanced stages, yellow-stippled and granular on the surface. In a cross-section, cortex and midzone were seen to be swollen. Microscopically, crystalline deposits, consisting of rosettes of yellow needles, were found in distal tubules, which appeared to accumulate proximally into Henle's loops and, in advanced cases, even into proximal tubules. Following administration of 2,8-dioxyadenine, only a few isolated bodies, the size of red cells, and staining bluish with hematoxylin, were found in distal tubules. Details of finer histological lesions will be reported separately.³

Oral Administration of Isotopic Adenine-Two male rats, weighing a

² The solubility of 2,8-dioxyadenine hemihydrate was found to be about 2 mg. per liter of water at room temperature. In comparison, uric acid has a solubility of about 55 mg. per liter.

³ Philips, F. S., Thiersch, J. B., and Bendich, A., in press.

total of 398 gm., received by stomach tube 190 mg. of adenine, containing 0.492 atom per cent excess N^{15} (14), prepared as a 2.5 per cent solution in 0.4 N lactic acid. The animals, kept on a Rockland stock diet (complete), were sacrificed 17 hours later, and the kidneys, which were much enlarged and showed typical "adenine kidney" lesions, were homogenized in dry ice-alcohol and dried with alcohol and ether. The kidney powder was extracted for 15 minutes at 100° with 15 ml. of 2 N HCl. The extract was neutralized with concentrated ammonia, and the precipitate was collected after chilling. The crude 2,8-dioxyadenine was purified by dissolving in hot 2 N HCl and then precipitating with concentrated ammonia; 32 mg. were obtained, which amounted to about a 14 per cent conversion from the ingested adenine. After three more reprecipitations, the 2,8-dioxyadenine, dried at room temperature in a vacuum desiccator over P_2O_5 , was found to contain 0.473 atom per cent excess N^{15} .

C₅H₅O₂N₅·½H₂O. Calculated, N 39.8; found, N 39.9

The ultraviolet spectrum was that of 2,8-dioxyadenine (11) (see Table II). From the dilution from 0.492 to 0.473 atom per cent excess N¹⁵ it may be calculated that there were about 20 mg. of free adenine available per kilo of body weight which had mixed with the ingested isotopic adenine.

Intraperitoneal Administration of Isotopic Adenine—Eight rats with an aggregate weight of 1520 gm. received for 4 days daily intraperitoneal injections of isotopic adenine (prepared as described above). A total of 528 mg. of adenine was injected. The animals were sacrificed on the 5th day. There was a considerable accumulation of ascitic fluid in the peritoneal cavity; otherwise the kidneys presented the same picture described above. The kidneys were processed as above and yielded 52 mg. of 2,8-dioxyadenine which contained 0.461 atom per cent excess N¹⁵. A sample was recrystallized from 2 N H₂SO₄.

 $C_5H_5O_2N_5\cdot\frac{1}{2}H_2O$. Calculated, N 39.8; found, N 39.7

 $C_4H_4O_2N_4 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$. Calculated, N 31.1, S 7.12; found, N 31.6, S 7.22

The remaining organs were pooled and were homogenized and dried with alcohol and ether. Sodium nucleate was prepared from the tissue powder. Adenine and guanine were isolated from the sodium nucleate according to procedures previously described (15). Urea, ammonia, and allantoin were isolated from the urine. The N¹⁵ content of the various constituents is listed in Table I.

• It was possible to demonstrate considerable amounts of free adenine in the urine by means of filter paper chromatography in several media. No 2,8-dioxyadenine, isoguanine, or 8-oxyadenine was seen, although by this technique a few micrograms of these compounds can be detected.

Comparison of Synthetic and Natural 2,8-Dioxyadenine—The identity of synthetic (11) and natural 2,8-dioxyadenine (from rat kidney) was established by determining the elementary composition, by comparing the ultraviolet absorption spectra and the mobility of samples on filter paper strips. As for the filter paper chromatography, the procedures and solvent systems described by Vischer and Chargaff (19) were utilized. The "spots" were made visible both by the HgS method (19) and by exposure to an ultraviolet lamp (Mineralight). In all cases, the "spots" coincided by both methods and revealed the presence of single components only. $R_{I\!\!P}$ values were computed by the usual procedure (20).

The comparison is given in Table II. The slight discrepancies in the E_M values listed in Table II are well within the experimental error encoun-

Table I

Administration of Isotopic Adenine
Adenine, 0 492 atom per cent excess N¹⁵.*

	Intraperitoneal	Oral
Assertance of the extension defects the desired material and the extension defect and the extens	alom per cent excess N18	alom per cent excess N15
2.8-Dioxyadenine, from kidney	0.461	0 473
Sodium nucleic acids	0 026	
Free nucleic acids	0 026	
Adenine	0 054	
Guanine sulfate	0 024	
Silver pyrimidines	0 004	
Ammonia	0 011	
Urea	0 008	

^{*} Consolidated-Nier ratio mass spectrometer, model 21-201; probable error ± 0.001

tered in such measurements with the Beckman quartz spectrophotometer, model DU (21).

Solubility of 2,8-Dioxyadenine in Water—About 5 mg. of 2,8-dioxyadenine hemihydrate were shaken at 23° with 10.0 ml. of water for 17 hours. The mixture was filtered and the filtrate was diluted with an equal volume of 2 n HCl. From the optical density (0.098) and the E_{M} of 17,700, it was calculated that the solubility of 2,8-dioxyadenine hemihydrate was about 2.0 mg. per liter of water.

To safeguard against the possibility that equilibrium might not have been reached, the experiment was repeated with these modifications. The suspension was heated for 15 minutes at 100° and then shaken at 25°

We are indebted to Dr. C. E. Carter, Oak Ridge National Laboratory, for placing this method at our disposal prior to publication.

for 2 days. The solubility was found to be about 2.2 mg. per liter of water.

Effect of Intraperitoneal Administration of Isoquanine (2-Oxyadenine)—Four rats, with an aggregate starting weight of 723 gm., were given daily injections by the intraperitoneal route for 3 days a total of 237 mg. of isoquanine (10) (0.5 per cent solution in 10 per cent gum acacia made up in 1 per cent NaCl, and containing 2 molar equivalents of lactic acid). The dose was equivalent to 100 mg. of adenine per kilo. The animals were sacrificed 1 day later, and their kidneys were removed. The peritoneal cavity contained considerable sterile ascitic fluid. The fluid was mixed

TABLE II
Comparison of Synthetic and Isolated 2,8-Dioxyadenine

Solvent	Wave- length	Ultraviolet	spectrum, E _M	
501.022	length	Synthetic	From kidney	
	mμ			
0.1 n phosphate, pH 6.8	235	8,890	8,950	
0.1 " " 6.8	305	15,400	15,500	
1 n HCl	305	17,400	17,700	

	Solvent system		Filter paper chromatography, Rp values		
		Synthetic	From kidney		
n-Butanol	(saturated with H ₂ O); NH ₂ atmosphere	0.01	0.01		
"	diethylene glycol, HCl	0.20	0.21		
"	" morpholine, H ₂ O; NH ₂ atmos-				
phere		0.24	0.24		
n-Butanol	l, diethylene glycol, H ₂ O; NH ₂ atmosphere	0.10	0.10		

with an equal volume of 2 n HCl, and the mixture was heated at 100° for 15 minutes and then filtered. The filtrate showed only non-specific ultraviolet absorption. Typical "adenine kidneys" were found. No other significant tissue changes were observed on microscopic examination. After freezing in dry ice-alcohol, the kidneys were homogenized in the Waring blendor and dried. The residue was extracted with 12 ml. of 2 n HCl at 100° for 20 minutes. The hot extract was neutralized with concentrated ammonia, cooled overnight, and the precipitate was collected. It was redissolved in acid, treated with norit, and reprecipitated. 75.1 mg. of a colorless solid, which amounted to a conversion of 26 per cent, were obtained. The preparation was recrystallized four times in this fashion and dried at room temperature in a vacuum desiccator over P₂O₅.

The ultraviolet absorption spectrum and filter paper chromatogram were that of 2,8-dioxyadenine. No evidence of the presence in the urine of 2,8-dioxyadenine or isoguanine could be obtained on examination by ultraviolet spectroscopy and by filter paper chromatography.

Intraperitoneal Administration of Isotopic Isoguanine—465 mg. of isoguanine sulfate hydrate (1.51 atom per cent excess N¹⁵ (10)) were dissolved in 4.88 ml. of 1 n NaOH and the volume was adjusted to 60 ml. by the addition of water. The solution was injected intraperitoneally once a day for 3 days into three male rats weighing a total of 872 gm. This level is equivalent to 100 mg. of adenine per kilo. The rats had received 58 ml. of the solution and were sacrificed on the 4th day. Except for the absence of ascites, the rats presented the same gross and microscopic anatomical picture as in the previous isoguanine experiment. The kidneys were processed as before and yielded 103 mg. of 2,8-dioxyadenine, which was identified spectroscopically. This amounts approximately to a 29 per cent conversion of the injected compound. The spectrum was that of 2,8-dioxyadenine.

Isotope analysis of the isolated compound, 1.48 atom per cent excess N¹⁵, indicated direct conversion of isoguanine to 2,8-dioxyadenine with a small but significant dilution by non-isotopic precursors.

To determine the extent of conversion of the isoguanine to allantoin, the following method for the determination of allantoin based upon the isotope dilution principle (22) was developed. A total of 133 mg. of urine plus washings (under toluene) was collected during the experimental period. To 50.0 ml. of this urine were added 10.1 mg. of analytically pure allantoin⁵ containing 0.602 atom per cent excess C¹³, and after complete solution and mixing, allantoin was isolated by a procedure previously described (15).

From the C¹³ content of the isolated allantoin, 0.085 per cent excess C¹³, it may be calculated that 160 mg. of allantoin were present in the urine collected during the course of the experiment. From this and from the N¹⁵ value of 0.054 atom per cent excess N¹⁵, one may calculate that only 1.3 per cent of the isoguanine injected had been converted into allantoin. Thus, only 3.6 mg. of allantoin had been derived directly from the administered isoguanine.

Duplicate samples of urea isolated from the urine as the dixanthydrol derivative (15) contained 0.011 and 0.013 atom per cent excess N¹⁶.

Oral Administration of Isotopic Isoguanine-Four male rats weighing

⁵ The isotopic allantoin was obtained from a biological experiment (Bendich, A., and Brown, G. B., unpublished results).

1217 gm. received during the course of 3 days 1.10 gm. of isoguanine sulfate hydrate (10), 5.40 atom per cent excess N¹⁵, admixed with moistened Rockland rat diet (complete). The animals, weighing 1268 gm., were sacrificed on the 4th day, and the nucleic acids of the mixed organs were isolated and worked up as described previously (15). Various nitrogenous constituents of the urine were isolated and examined, and the results are listed in Table III.

The results show that isoguanine does not function as an effective precursor for nucleic acid purines when furnished orally to rats (16). If the assumption is made that the excretion of allantoin in this experiment was the same as in the previous experiment, it may be calculated that only about 7 per cent of the ingested isoguanine had been converted to allantoin.

TABLE III
Feeding of Isotopic Isoguanine*

	Atom per cent excess N ¹¹
Isoguanine	5.40
Nucleic acids	
Purine hydrochlorides	0.007
Allantoin	
Urea	0.052
Total urinary nitrogen	

^{* 230} mg. of isoguanine per day for 3 days.

Intraperitoneal Administration of 8-Oxyadenine to Rats—Three rats totaling 603 gm. in weight received for 3 days daily intraperitoneal injections of a solution of 8-oxyadenine (11). The compound (290 mg.) was made up in 52 ml. of solution containing 2 molar equivalents of lactic acid, 5 gm. of gum acacia, 500 mg, of NaCl, and 1 drop of caprylic alcohol. In all, 196 mg. of 8-oxyadenine were injected, a level equivalent to 100 mg. of adenine per kilo per day. The animals were sacrificed on the 4th day, and the kidneys, showing typical "adenine kidney" lesions, were frozen in dry ice-alcohol, homogenized, and dried. Considerable sterile ascitic fluid had accumulated in the peritoneal cavity, but the fluid revealed no characteristic, specific absorption when examined by ultraviolet spectroscopy. The kidneys were processed as described above and 116 mg, of 2.8-dioxyadenine were obtained; this indicated a conversion of 36 per cent. For analysis, the six times recrystallized product was dried at room temperature in a vacuum desiccator over P₂O₅. The spectrum was that of 2,8-dioxyadenine

No 8-oxyadenine or 2,8-dioxyadenine could be demonstrated in the urine by ultraviolet spectroscopy or by filter paper chromatography.

Oral Administration of 2,8-Dioxyadenine—Three rats weighing 758 gm. were given by stomach tube a total of 335 mg. of 2,8-dioxyadenine (11) suspended in 15 ml. of 10 per cent gum acacia. The animals were kept on a normal diet and were sacrificed 17 hours later. Urine and feces were collected. The kidneys appeared normal. They were extracted with 2 n HCl as described above, but the extracts yielded no 2,8-dioxyadenine and showed no specific ultraviolet absorption. No 2,8-dioxyadenine could be demonstrated in the viscera and only traces could be found in the urine. However, a hot 2 n HCl extract of the feces revealed that about 10 to 20 per cent of the dietary dioxyadenine had been eliminated presumably unabsorbed.

Intrapersioneal Administration of 2,8-Dioxyadenine-A total of 371 mg. of 2,8-dioxyadenine hemihydrate was injected into four rats intraperitoneally (weighing 757 gm.) over a period of 4 days. The compound was made up in 60 ml. of solution, containing 10 per cent gum acacia, 1 per cent NaCl, and 2 equivalents of lactic acid. The animals were sacrificed on the 5th day. There was considerable accumulation of sterile ascitic fluid in the peritoneal cavity, but no 2,8-dioxyadenine could be demonstrated in acidic extracts by ultraviolet spectroscopy. The kidneys appeared normal. When the kidneys were extracted with 2 N HCl and the extract neutralized with ammonia, no 2,8-dioxyadenine precipitated and no spectrum corresponding to this substance was obtained. It would have been possible to demonstrate the presence of 10 γ or less if it were present. Examination of 2 N HCl extracts of the organs likewise revealed no 2,8-dioxyadenine. When the urine was examined by filter paper chromatography in various media and by ultraviolet spectroscopy, no evidence for the presence of more than mere traces of 2.8-dioxyadenine could be obtained.

The authors wish to express their appreciation to Mrs. Helen Getler, Mrs. Marie Borgatta, and Mr. William D. Geren for assistance, to Mr. Roscoe C. Funk, Jr., for the microanalyses, and to Dr. Harold Beyer and Mr. John Deonarine for cooperation in the isotope determinations.

SUMMARY

The deposition of 2,8-dioxyadenine in the kidneys of the rat following the administration of adenine has been studied. The identity of the product has been confirmed. Evidence that 2,8-dioxyadenine arises as a result of a direct *in vivo* oxidation of adenine is presented. The resulting lesion has been described and has been termed "adenine kidney."

2,8-Dioxyadenine also deposits in rat kidneys when either isoguanine or 8-oxyadenine is administered, but none could be demonstrated after the administration of 2,8-dioxyadenine itself.

In connection with the biosynthesis of nucleic acids, it was found that intraperitoneally injected adenine serves as a precursor of nucleic acid adenine and guanine, but that isoguanine is inactive in this regard.

BIBLIOGRAPHY

- 1. Minkowski, O., Arch. exp. Path. u. Pharmakol., 41, 375 (1898).
- 2. Nicolaier, A., Z. klin. Med., 45, 359 (1902).
- 3. Ebstein, W., and Bendix, E., Virschows Arch. path. Anat., 178, 464 (1904).
- 4. Fischer, E., Ber. chem. Ges., 30, 2226 (1897).
- 5. Raska, S. B., J. Am. Med. Assn., 131, 1093 (1946).
- 6. Raska, S. B., J. Biol. Chem., 165, 743 (1946).
- 7. Purrmann, R., Ann. Chem., 544, 182 (1940).
- 8. Cherbuliez, E., and Bernhard, K., Helv. chim. acta, 15, 464, 978 (1932).
- 9. Spies, J. R., J. Am. Chem. Soc., 61, 350 (1939).
- 10. Bendich, A., Tinker, J. F., and Brown, G. B., J. Am. Chem. Soc., 70, 3109 (1948).
- 11. Cavalieri, L. F., and Bendich, A., J. Am. Chem. Soc., in press.
- 12. Coombs, H. I., Biochem. J., 21, 1259 (1927).
- 13. Booth, V. H., Biochem. J., 32, 494 (1938).
- 14. Cavalieri, L. F., Tinker, J. F., and Bendich, A., J. Am. Chem. Soc., 71, 533 (1949).
- Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., J. Biol. Chem., 172, 469 (1948).
- 16. Brown, G. B., Cold Spring Harbor Symposia Quant. Biol., 13, 43 (1948).
- 17. Bendich, A., and Brown, G. B., J. Biol. Chem., 176, 1471 (1948).
- 18. Wintrobe, M. M., Clinical hematology, Philadelphia, 2nd edition, 449 (1946).
- 19. Vischer, E., and Chargaff, E., J. Biol. Chem., 176, 703 (1948).
- 20. Consden, R., Gordon, A. H., Martin, A. J. P., Biochem. J., 38, 224 (1944).
- Cavalieri, L. F., Bendich, A., Tinker, J. F., and Brown, G. B., J. Am. Chem. Soc., 70, 3875 (1948).
- 22. Rittenberg, D., and Foster, G. L., J. Biol. Chem., 133, 737 (1940).

THE COMPARATIVE AVAILABILITIES OF MIXTURES OF THE L AND DL MODIFICATIONS OF THE ESSENTIAL AMINO ACIDS FOR GROWTH IN THE RAT*

By JOHN F. VAN PILSUM AND CLARENCE P. BERG

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

(Received for publication, September 12, 1949)

It has heretofore frequently been assumed, though never established, that growth on an amino acid mixture may be adversely affected by the use of racemic, instead of natural, amino acids in the mixture. This was first suggested by Albanese and Irby who surmised that weight losses which they noted in young rats fed an essential amino acid mixture containing several DL-amino acids might be attributable at least in part to the "toxic effects of unnatural forms of certain amino acids that can not be utilized" (1). Their experimental results have not been confirmed. The consistency with which growth has been obtained in analogous tests before (2, 3) and since (4-7) leaves little doubt that the growth failure which they observed must have been due primarily to some other factor, probably to an isoleucine deficiency (8, 6).

Confusion has resulted from the use in most of the growth tests thus far carried out of control diets which have contained casein (4, 5, 7) or casein hydrolysates (1, 4). These have afforded better growth than have the essential amino acid mixtures. Whether the difference may be ascribed exclusively to the presence in the casein or casein hydrolysates of the unessential amino acids, which are known to accelerate growth on essential amino acid mixtures (3, 6), or may result in part from growth retardation by the DL-amino acids in the essential amino acid mixture can only be guessed.

Recently Wretlind (7) has reported that, at dietary levels of 20 to 30 per cent, mixtures of the DL modifications of the nine essential amino acids (arginine excluded) produced less rapid growth than at lower levels, hence may have been toxic. Growth retardation was also noted when only one of the DL-amino acids was greatly increased. The possibility that similar tests with L-amino acids might have produced similar results was not considered. In comparative growth tests of mixtures containing both the es-

^{*}The data in this paper were taken largely from a dissertation submitted by John F. Van Pilsum in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

The studies were made possible by a generous grant-in-aid from The Dow Chemical Company of Midland, Michigan.

sential and non-essential amino acids with casein, Ramasarma, Henderson, and Elvehjem (9) noted slower growth on the former. They suggest that at the levels used "the presence of the unnatural p-isomers" in the mixtures "may not produce any symptoms of toxicity but may cause a slight depression of growth," also that "an imbalance of the amino acids in the mixtures employed" might be involved.

The purpose of this communication is to present the results of tests in which growth on mixtures of the ten essential amino acids in the DL form has been compared directly with growth on similar mixtures containing only the corresponding L modifications. So far as we are aware, such studies have not previously been reported, nor indeed have mixtures containing only the natural forms of the essential amino acids ever heretofore been employed.

EXPERIMENTAL

Most of the DL-amino acids used in these studies were made available to us by The Dow Chemical Company.¹ The DL-arginine monohydrochloride monohydrate was prepared from gelatin by the procedure of Schein and Berg (10). Part of the DL-threonine was synthesized and freed of allothreonine as outlined by West and Carter (11). The remainder was purchased from Merck and Company, as was also the DL-isoleucine.² Most of the DL-histidine was purchased as the monohydrochloride dihydrate from the Nutritional Biochemical Corporation. All of the DL-amino acids were recrystallized at least twice. The tryptophan, lysine monohydrochloride, histidine monohydrochloride dihydrate, and arginine monohydrochloride monohydrate were analyzed for amino nitrogen by the Van Slyke method, the methionine, phenylalanine, valine, leucine, threonine, and isoleucine for total nitrogen by Kjeldahl. In every instance the determined values agreed well with the calculated.

Table I affords data pertaining to the optical rotations of the various samples of the L-amino acids employed. It also indicates the method of resolution or isolation of the lots prepared by us and the sources of those obtained elsewhere.

Table II gives the composition of the L and the DL mixtures employed initially in the feeding tests. The L mixture afforded twice the minimal amounts of the essential amino acids tentatively indicated by Rose (23) to

¹We wish to thank Dr. L. S. Roehm and The Dow Chemical Company for this generosity and the cooperation extended us on several occasions.

²Dr. E. E. Howe of Merck and Company, Inc., has assured us that the pr-threonine is free of allothreonine, as judged by microbiological assay and solubility data, and that the pr-isoleucine is 97 per cent pure, and possibly of even higher purity.

	[4	[a] _D		ntration per 100 ml. solution	Solvent	Temper- ature	graphic
Amino acid	Found	Recorded in reference	As read	Recorded in reference	used	of solution read	refer- ence No.
	degrees	degrees	gm.	gm.		°C.	
Methionine, Lot 1	-7.3	-7.5	1.0	0.8	H ₂ O	25	(12)
" 2*	-7.2		1.0		"	25	
Phenylalanine, Lot 1	-34.0	-35.14	1.0	1.9	"	20	(13)
" " 2†	-32.0		1.0		"	25	
Tryptophan, Lot 1	-32.0	-32.1	0.5	0.5	"	20	(14)
" " 2‡	-31.0		0.5		"	20	(15)
Histidine monohydro- chloride monohy- drate‡	1 .	+8.0	1.0	Not recorded	3 m HCl	25	(16)
Arginine monohydro-	+22.0				n HCl	25	1
chloride§	+12.0	+12.3	5.0	5.0	H ₂ O	25	(17)
Lysine monohydro- chloride, Lot 1§	+20.0		1.0		м HCl	26	
Lysine monohydro- chloride, Lot 2‡	+20.1		1.0		"	26	(18)
Lysine monohydro- chloride, Lot 3	+21.0		1.0		"	26	(19)
Valine	+29.0	+28.7	1.0	3.56	20% HCl	20	(20)
Leucine	+15.1	+15.8	1.0	4.54	20% "	20	(21)
Isoleucine	+40.0	+40.6	1.0	5.09	20% "	20	(22)
Threonine, Lot 1	-28.0	-28.3	1.0	Not recorded	H ₂ O	25	(11)
" " 2*	-28.0		1.0		"	25	

Table I
Specific Rotations of L-Amino Acid Preparations Used in Feeding Tests

^{*} We are happy to acknowledge our indebtedness to Dr. J. P. Greenstein of the National Cancer Institute who so kindly volunteered to send us a generous supply of L-methionine, and to Dr. E. E. Howe of the Research Laboratories of Merck and Company who so graciously provided us with the L-threonine needed to continue our tests.

[†] This lot of L-phenylalanine was purchased from the Nutritional Biochemical Corporation.

[‡] L-Histidine monohydrochloride monohydrate and the samples of tryptophan and lysine monohydrochloride thus indicated were prepared in the laboratory, the first from spray-dried blood, the last two from casein by the method indicated in the reference. Application of Sullivan's naphthoquinone test indicated that the histidine monohydrochloride was free of cystine.

[§] The arginine monohydrochloride and one of the samples of lysine monohydrochloride were purchased from Merck and Company.

^{||} These samples were prepared through the dihydrochlorides, both of which showed specific rotations of 15.0° when 3 gm. were dissolved in water, made up to 100 cc., and polarized at 20°. The literature records 15.6° (19).

be necessary to support normal growth when the non-essential amino acids are also supplied. The DL-I mixture contained the same quantities of the L-amino acids, though supplied as components of the DL modifications. The DL-II mixture provided half as much DL-arginine, tryptophan, phenylalanine, and methionine as the DL-I mixture, and an intermediate level of DL-histidine, but the same amounts of the other five amino acids.

TABLE II
Composition of Amino Acid Mixtures

	ı	DL-I	DL-II
	gm	gm.	gm.
Valine	1 4	2.8	2.8
Leucine	1 6	3.2	3 2
Isoleucine	1 0	2 0	20
Lysine* .	2 0	4.0	4.0
Threonine	1 0	2.0	2.0
Tryptophan	0 4	0.8	0.4
Methionine	1 2	2.4	1.2
Phenylalanine	1 4	2.8	1 4
Histidine*	0.8	1.6	1.2
Arginine*	0 4	0 8	0.4
	11 2	22 4	18.6
HCl and H ₂ O combined with amino acids*	0 86	1 89	1.63
NaHCO ₂ added*	1 75	4 52	4 12
	13 81	28.81	24.35

^{*} The L-lysine and the L- and DL-arginine were fed as the monohydrochlorides, the L-histidine as the monohydrochloride monohydrate, the DL-histidine as the monohydrochloride dihydrate, and the DL-lysine usually as the monohydrochloride. The NaHCO₂ was equivalent to the HCl combined with the amino acids fed. When the DL-lysine was fed as the dihydrochloride, the extra HCl and NaHCO₂ added 2.3 gm. to the total weights of the DL-I and DL-II mixtures.

The two isomers of tryptophan (24, 25, 6), methionine (26, 2), and phenylalanine (27) show nearly, if not quite the same, growth-promoting capacities. The indispensability of arginine becomes evident only in diets affording maximal growth (28). The D form of histidine promotes growth, but less rapidly than the L (29). On the other hand, only the L modifications of lysine (30), threonine (31), and valine, leucine, and isoleucine (2) possess appreciable growth-promoting capacities. Theoretically, therefore, the DL-II mixture should have possessed approximately the same basic growth-promoting potentialities as the L mixture.

In the first of the five series of tests conducted, each of the three amino acid mixtures was incorporated in a diet similar to that which we have heretofore commonly employed (32), except that it contained the salt mixture of Jones and Foster (33), 26 per cent of hydrogenated cottonseed oil, and dextrin instead of starch. Nicotinic acid and folic acid were included in the vitamin supplement, in addition to the factors noted in the reference cited. Subsequent trial of the low fat type of diet employed by Rose, Oesterling, and Womack (6) proved it to be so superior that its use was adopted. Hence, in all of the other tests, each 100 gm. of diet contained, in addition to the amino acid mixture (or other source of protein nitrogen), corn oil 2.0, vitamin A and D concentrate³ 0.08, salt mixture (33) 4.0, Cellu flour 2.0, inositol 0.1, choline chloride 0.2, liver extract 0.4,4 vitamin B₁₂ concentrate⁵ 0.1 gm., and dextrin to make 100 gm. To each kilo of ration were added thiamine hydrochloride 5.0, riboflavin 10.0, pyridoxine hydrochloride 5.0, nicotinic acid 5.0, calcium pantothenate 25.0, p-aminobenzoic acid 300.0, α-tocopherol 25.0, 2-methyl-1,4-naphthequinone 2.0, biotin 0.1, and folic acid 0.1 mg.

Weanling rats of the Sprague-Dawley strain were used throughout. They were housed individually and allowed continual access to food and water. The test periods were 28 days long.

Table III shows the comparative growth responses obtained on the high fat and low fat types of diet. In the animals fed the high fat diet (Series I), growth on the DL-II mixture exceeded that on the L mixture. In the rats fed the low fat diet (Series II), growth on these mixtures was approximately the same, though inferior to that on a casein hydrolysate. Growth on the DL-I mixture was definitely inferior in both series to that on either the L or the DL-II mixture. No gross abnormality was detectable on postmortem examination of the animals fed the L and the DL-II mixtures, but all of the animals fed the DL-I mixtures showed darkened spleens.

Table IV summarizes data which indicate (Series III) that the agent chiefly responsible for the growth retardation on the DL-I mixture was the extra DL-methionine which it contained. In contrast with the marked

⁸Oleum percomorphum, marketed by Mead Johnson and Company. Per gm. it contained 60,000 U. S. P. units of vitamin A and 8500 U. S. P. units of vitamin D.

⁴Liver concentrate 1:20, kindly supplied by The Wilson Laboratories, through the courtesy of Dr. S. W. Hier.

⁶We are indebted to Dr. L. R. Hines and E. R. Squibb and Sons for a generous supply of Preparation ER-72-4, which contained 15 γ of vitamin B₁₂ per gm., with starch as the diluent.

⁶This was an acid hydrolysate made available to us by The Upjohn Company, through the courtesy of Dr. John T. Correll and Dr. Curtis E. Meyer. It was fortified as indicated in Table III.

TABLE III

Growth in 28 Days on Diets Containing Essential Amino Acid Mixtures Composed

Entirely of L- or Entirely of DL-Amino Acids

Series No.	No. of rats	Basal diet*	Gain in weight	Food consumed
	-		gm.	gm.
I	3♂,3♀	L	33 ± 11	101 ± 11
	2♂,3♀	DL-I	28 ± 6	87 ± 11
	3♂,3♀	DL-II	49 ± 11	118 ± 20
II	20,29	L	80 ± 10	244 ± 26
	5♂,5♀	DL-I	44 ± 12	137 ± 27
	70,79	DL-II	76 ± 8	217 ± 19
	3♂,3♀	CH-11.7	99 ± 9	288 ± 32

The basal diet used in Series I was high in fat, that in Series II was low. L indicates 11.2 per cent of L-amino acids, DL-I 22.4 per cent of DL-amino acids, and DL-II 18.6 per cent of DL-amino acids. CH-11.7 indicates a diet which contained 11.7 per cent of an acid hydrolysate of casein, instead of a mixture of purified amino acids. The hydrolysate was fortified with 0.6 gm. of DL-methionine and 1.1 gm. of DL-tryptophan per 100 gm., and was isonitrogenous with the L-amino acid mixture.

Table IV

Growth Retardation Induced in 28 Days by Supplementing DL Mixtures of Essential

Amino Acids with Additional DL-, L-, or D-Methionine

Series No.	No. of rats	Basal diet	Supplement per 100 gm. diet	Gains in weight	Food consumption
			gm.	£m.	gm.
III	1♂,4♀	DL-II	None	70 ± 8	208 ± 20
	20,39	"	DL-Histidine 0.4	68 ± 4	195 ± 11
	30,20	"	DL-Tryptophan 0.4	65 ± 9	214 ± 26
	30,22	"	DL-Arginine 0.4	73 ± 3	211 ± 13
	3♂,2♀	"	DL-Phenylalanine 1.4	73 ± 11	219 ± 15
	20,39		DL-Methionine 1.2	41 ± 11	152 ± 10
	20	"	All of above*	40 ± 6	146 ± 6
IV	50	**	None	80 ± 4	233 ± 5
	5♂	"	DL-Methionine 1.2	68 ± 8	185 ± 9
	5♂	"	L-Methionine 1.2	48 ± 6	144 ± 14
	50	"	p-Methionine 1.2	71 ± 13	189 ± 14
	507	"	DL-Histidine 0.4, arginine	100 ± 10	268 ± 10
			0.4, tryptophan 0.4, and phenylalanine 1.4		
	5♂	"	Same + DL-methionine 1.2*	56 ± 6	168 ± 10
	5♂	CH-11.7†	None	92 ± 12	275 ± 19
	50	"	DL-Methionine 0.6	81 ± 15	236 ± 50
	5♂	"	" 1.8	40 ± 6	144 ± 24
	5♂	"	" 3.0	13 ± 7	109 ± 11

^{*} The supplements indicated converted the DL-II into the DL-I mixture.

[†] Per 100 gm., this diet contained 11.7 gm. of an acid hydrolysate of casein which had been fortified as indicated in Table III.

growth retardation induced upon increasing the DL-methionine to the level fed in the DL-I diet, variations in growth induced by increasing the other amino acids individually to the levels prevailing in the DL-I mixture were minor and of doubtful significance.

The data in Series IV show that the simultaneous addition to the DL-II mixture of 0.4 gm. each of DL-histidine, tryptophan, and arginine and 1.4

TABLE V

Series V, Growth in 28 Days on L and DL Mixtures of Essential Amino Acids Made

Isonitrogenous by Addition of Glycine and Ammonium Citrate

No. of	Basal diet	Supplement per 100 gm. diet	Gains in weight	Food consumption	
		gm.	gm.	gm.	
3♂	L-a*	None	97 ± 3	263 ± 16	
3♂	"	Glycine 3.7 and NH ₄ citrate 2.9†	97 ± 11	254 ± 36	
5♂	DL-II-a*	None	97 ± 11	247 ± 25	
40	"	Glycine 3.7 and NH ₄ citrate 2.9	93 ± 15	258 ± 26	
ර්ථ	"	DL-Phenylalanine 0.8, histidine 0.4, tryptophan 0.4, and arginine 0.4‡	100 ± 16	270 ± 30	
4♂	CH-12.5§	None	106 ± 9	310 ± 19	
5♂	"	DL-Methionine 2.0	63 ± 8	200 ± 22	
5ਹੋ	4.6	2.0, glycine 2.0, and L-arginine 1.0	97 ± 19	265 ± 39	
3♂	CH-20.6§	None	159 ± 4	362 ± 18	
3♂	CH-28.7§	"	158 ± 4	381 ± 13	

^{*} The L-a diet contained 0.6 gm. more L-phenylalanine per 100 gm. than the L diet, the DL-II-a diet 0.6 gm. more DL-phenylalanine than the DL-II.

gm. of phenylalanine increased the rate of growth significantly. They also indicate that the growth retardation induced by the L isomer of methionine was significantly greater than that induced by either the DL or the D form. Supplementary tests with casein hydrolysate showed that as methionine was added in increasing quantities the capacity of the diet to promote growth was progressively impaired. Darkened spleens were found only in the animals fed the DL-II mixtures supplemented with methionine and in the animals fed diets containing the hydrolysate to which 1.8 gm. or more of methionine had been added per 100 gm. of diet.

[†] These supplements compensated for the extra nitrogen supplied in the DL-II-a mixture.

[‡] The DL-II-a diet plus these supplements was identical to the DL-II diet plus the supplements of these four amino acids used in Series IV (Table IV).

[§] The hydrolysate was from the lot used also in Series II and Series IV. At the 12.5 per cent level, the diet containing it was isonitrogenous with the L-a diet, at the 20.6 per cent level with the DL-II-a diet, and at the 28.7 per cent level with the DL-II-a diet which had been supplemented with the glycine and the ammonium citrate.

In Series V (Table V), the phenylalanine content of each of the amino acid mixtures used was increased by 0.6 gm. per 100 gm. of diet. Critical examination of the rates of growth on the L-a and the DL-II-a diets reveals little or no difference. The extra nitrogen supplied some of the animals as glycine and ammonium citrate neither enhanced nor significantly retarded the growth rate.

Gains on the L-a and the DL-II-a mixtures approximated those on the diet which contained 12.5 per cent of the casein hydrolysate, but it should be noted that at that level the hydrolysate may not have provided enough tryptophan for optimal growth response. Gains on the diet containing 12.5 per cent of the hydrolysate were considerably less than those on diets containing 20.6 and 28.7 per cent. Incidentally, growth on the latter equaled that attained on a stock diet of Purina laboratory chow.

Unrecorded paired feeding tests indicated that restriction of the food intake was probably largely responsible for the slower growth of the animals receiving the 2 per cent supplement of DL-methionine, but not for the darkened spleens. Supplementation of the high methionine type of diet with glycine and L-arginine stimulated food consumption and growth, but did not quite prevent the spleen darkening. Preliminary tests have indicated that the addition of 1 and 2 per cent of choline to the CH-12.5 diet produces darkening of the spleen, but does not promote marked growth retardation.

DISCUSSION

The data obtained seem to us to prove rather conclusively that in our tests the D components of the DL mixtures exerted no appreciable deleterious effect *per se*, despite the fact that they were present in amounts ranging from 9.3 to 10.6 per cent of the various diets.

The growth-retarding effect in the rat of relatively high levels of plemethionine has been previously noted. Kade and Sheperd (34) observed that the addition of 1 per cent of plemethionine afforded increased utilization of an 8 per cent casein diet, that 1.5 per cent promoted no enhancement, and that 2 to 3 per cent was inhibitory. Brown and Allison have reported marked weight losses on diets containing 12 per cent of casein and 4.8 per cent of plemethionine (35). In tests of the plements of the essential amino acids as dietary supplements, Wretlind (7) has noted that plemethionine can be increased the least of all without causing retarded growth. Howe et al. (36) have found it the most poorly tolerated of the racemic amino acids upon intravenous infusion into dogs.

The observation (Series IV, Table IV) that the growth retardation induced by increasing the p-methionine content of the diet was appreciably less than that caused by adding the natural L isomer is of particular in-

terest. It is inconsistent with the supposition that the **D** forms of the essential amino acids are the more likely to cause "toxic effects" or depression of growth (1, 7, 9), especially if they are unable to promote growth (1). Smaller growth retardation by **D**-methionine may result from its more ready escape into the urine.

Histological examinations were made of the spleens of the animals on the various diets, with use of hematoxylin-eosin and iron stains. The kidneys, livers, lymph nodes, and bone marrow of about half of each group were also examined. We are greatly indebted to Dr. J. R. Carter of the Department of Pathology for making these studies. He reports finding no significant abnormalities in any organ except the spleen. Detailed cellular counts of the bone marrow were not made, but no striking abnormalities were detected. In all of the animals fed the higher levels of DL-, D-, or L-methionine, the spleen showed varying degrees of congestion, with formation of hemosiderin pigment. The normal architectural pattern was well preserved, and no significant cellular changes were observed. No histological differences were noted between animals fed the L- and those fed the DL-amino acid mixtures with the lower level of methionine. Hemosiderin was not found in their spleens in significant amounts.

The reason for the findings on the spleens of the rats fed the higher levels of methionine is uncertain. Examinations of the cellular components of the blood have thus far afforded no suggestive clues beyond a low leucocyte count. Reticulocyte and erythrocyte counts, hemoglobin (Newcomer), and fragility tests have shown no marked divergencies.⁷ The fact that spleen darkening is induced also by feeding excess choline seems to indicate that labile methyl groups may be at least partially responsible. Possibly alleviation of the condition upon the addition of glycine and arginine to the diets containing excess methionine (Table V) may reflect the conversion of such groups to creatine. In studies made in the chick, McKittrick (37) noted growth depression upon feeding excess methionine, but none when choline chloride was fed, unless the diet was supplemented with homocystine. Counteraction of the growth-depressing effect was observed upon adding glycocyamine or serine. In the rat, Brown and Allison (35) noted less growth retardation and an increased creatinine excretion when L-arginine was added to their high methionine diets. The growth retardation noted in our studies was not altered by doubling the vitamin allotment (vitamin B₁₂ excepted).

Rose has reported growth enhancement upon supplementing diets containing 5.8 and 2.8 per cent of only the essential amino acids with glycine and ammonium citrate (38). It seems probable that our diets may already have provided as much extra nitrogen as the organism could use for

⁷The cooperation of Mr. Wm. A. Phillips who made the blood tests is appreciated.

the synthesis of unfurnished amino acids. Some justification for assuming that the extra nitrogen might have stimulated growth, had our mixtures been fed at lower levels, is found in the better growth in Series I (Table III) of the rats fed the DL-II mixture than in those receiving only the L-amino acids. In Series I the food consumption, hence the amino acid intake, was much smaller than in the subsequent tests. Little information is available concerning the minimal or optimal proportions of the essential amino acids for promoting growth, when the essential amino acids constitute the sole source of protein nitrogen. We have some evidence that our original DL-II and L mixtures may have contained more threonine than necessary. On the other hand, they may have contained too little phenylalanine and possibly border line amounts of some of the other amino acids.

SUMMARY

Rats fed the L forms of the ten essential amino acids as components of a DL mixture constituting 22.4 per cent of the diet grew less well than control rats fed only the L isomers at a dietary level of 11.2 per cent. When allowance was made for the growth-promoting capacities of the D components of the DL mixture, and only half as much DL-phenylalanine, tryptophan, methionine, and arginine and an intermediate level of DL-histidine were included, the resulting 18.6 per cent of DL-amino acids promoted as good growth as that attained on the L mixture.

By adding each of the lowered amino acids in turn to the 18.6 per cent DL diet, the growth retardation was traced to excess methionine. Removal of half of the DL-methionine completely overcame the growth-retarding effect of the 22.4 per cent DL mixture. Comparative tests showed that the growth retardation produced by the natural L isomer of methionine was greater than that produced by either the DL or the D modification.

No differences were noted in comparative tests of the blood or in postmortem examination of the rats fed the L and DL mixtures which did not contain excessive amounts of methionine. On the other hand, in the rats fed excessive amounts of methionine, either in amino acid mixtures or as supplements in diets containing casein or casein hydrolysates, the spleens were dark and were congested with hemosiderin pigment. Additions of glycine and arginine overcame the growth retardation, but not entirely the spleen congestion. Diets containing casein hydrolysates to which 1 and 2 per cent of choline were added produced darkened and congested spleens, but did not retard the growth. An extension of these tests is planned to determine to what extent labile methyl groups may be involved.

Increasing the phenylalanine contents of the 11.2 per cent L- and the 18.6

per cent decided by 0.6 per cent seemed to improve the growth promotion by each to about the same extent. Addition to these diets of extra nitrogen in the forms of glycine and ammonium citrate did not further enhance, nor did it retard, the growth. Possible reasons are suggested.

It seems fair to conclude that, contrary to the often repeated conjecture that the D forms of the essential amino acids may be toxic in the rat, proportionately large amounts can actually be fed as components of DL-amino acid mixtures without producing any apparent growth-retarding or other deleterious effect. Moreover, when fed in excessive amounts, the L form of an amino acid may, as has been shown in the case of methionine, be even more deleterious than its D modification.

BIBLIOGRAPHY

- 1. Albanese, A. A., and Irby, V., Science, 98, 286 (1943).
- 2. Rose, W. C., Physiol. Rev., 18, 109 (1938).
- 3. Bauer, C. D., and Berg, C. P., J. Nutr., 26, 51 (1943).
- 4. Martin, G. J., Proc. Soc. Exp. Biol. and Med., 55, 182 (1944).
- 5. Kinsey, V. E., and Grant, W. M., Science, 99, 303 (1944).
- 6. Rose, W. C., Oesterling, M. J., and Womack., M., J. Biol. Chem., 176, 753 (1948).
- 7. Wretlind, K. A. J., Acta physiol. Scand., 15, 304 (1948); 17, suppl. 59 (1949).
- 8. Berg, C. P., Ann. Rev. Biochem., 13, 239 (1944); Nutr. Rev., 3, 31 (1945).
- Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., J. Nutr., 38, 177 (1949).
- 10. Schein, A. H., and Berg, C. P., Arch. Biochem., 11, 215 (1946).
- 11. West, H. D., and Carter, H. E., J. Biol. Chem., 119, 109 (1937).
- 12. Windus, W., and Marvel, C. S., J. Am. Chem. Soc., 53, 3490 (1931).
- 13. du Vigneaud, V., and Meyer, C. E., J. Biol. Chem., 98, 295 (1932).
- 14. Berg, C. P., J. Biol. Chem., 100, 79 (1933).
- 15. Cox, G. J., and King, H., Org. Syntheses, 10, 100 (1930).
- 16. Foster, G. L., and Shemin, D., Org. Syntheses, coll. 2, 330 (1943).
- 17. Brand, E., and Sandberg, M., Org. Syntheses, coll. 2, 49 (1943).
- 18. Cox, G. J., King, H., and Berg, C. P., J. Biol. Chem., 81, 755 (1929).
- 19. Berg, C. P., J. Biol. Chem., 115, 9 (1936).
- 20. Fischer, E., Ber. chem. Ges., 39, 2320 (1906).
- 21. Fischer, E., and Warburg, O., Ber. chem. Ges., 38, 3997 (1905).
- 22. Locquin, R., Bull. Soc. chim., 1, 595 (1907).
- Rose, W. C., Science, 86, 298 (1937). Womack, M., and Kade, C. F., Jr., in Sahyun, M., Outline of the amino acids, New York, 229 (1944).
- du Vigneaud, V., Sealock, R. R., and Van Etten, C., J. Biol. Chem., 98, 565
 (1932).
- 25. Berg, C. P., J. Biol. Chem., 104, 373 (1934).
- 26. Jackson, R. W., and Block, R. J., J. Biol. Chem., 122, 425 (1937-38).
- 27. Rose, W. C., and Womack, M., J. Biol. Chem., 166, 103 (1946).
- Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M., and Rose, W. C., J. Biol. Chem., 166, 585 (1946).
- 29. Cox, G. J., and Berg, C. P., J. Biol. Chem., 107, 497 (1934).

- 30. Berg, C. P., J. Nutr. 12, 671 (1936).
- 31. West, H. D., and Carter, H. E., J. Biol. Chem., 122, 611 (1937-38).
- 32. Kallio, R. E., and Berg, C. P., J. Biol. Chem., 181, 333 (1949).
- 33. Jones, J. H., and Foster, C., J. Nutr., 24, 245 (1942).
- 34. Kade, C. F., Jr., and Sheperd, J., Federation Proc., 7, 291 (1948).
- 35. Brown, J. H., and Allison, J. B., Proc. Soc. Exp. Biol. and Med., 69, 196 (1948).
- Howe, E. E., Unna, K., Richards, G., and Seeler, A. O., J. Biol. Chem., 162, 395 (1946).
- 37. McKittrick, D. S., Arch. Biochem., 15, 133 (1947).
- 38. Rose, W. C., Federation Proc., 8, 546 (1949).

THE EFFECT OF PREVIOUS CARBOHYDRATE DEPRIVATION ON THE SYNTHESIS OF ALANINE BY LIVER IN VITRO*

By ATTILIO CANZANELLI, DAVID RAPPORT, AND RUTH GUILD (From the Department of Physiology, Tufts College Medical School, Boston)

(Received for publication, August 1, 1949)

While studying the influence of the hormones on alanine synthesis by the liver, we observed that occasionally our normal control animal gave an unusually low value, which seemed to be associated with a low intake of food the previous day. That the composition of the diet might affect the activity of a tissue has been repeatedly observed. Miller (1), Potter and Klug (2), Kaplan and Greenberg (3), and Seifter, Harkness, Feldman, Rubin, and Muntwyler (4) all have presented evidence that fasting or the administration of modified diets for long periods affected the enzymatic activity of tissue. Deamination of alanine by mouse liver slices was found by Kaplanskii, Berezovskaya, and Shmerling (5) to be completely stopped when the animals were subjected for 20 to 35 days to a diet low in protein. Neber (6) observed that liver slices of guinea pigs fasted for 2 days produced less amino nitrogen from pyruvic acid and ammonium carbonate. In view of the many mechanisms that might be involved, we decided to study the effect of fasting and of variation in composition of the diet on the synthesis of alanine by rat liver slices.

Methods

Adult rats of the Wistar strain were given the following basic diet for five days: casein 25 per cent, sucrose 50 per cent, Crisco 20 per cent, salt mix 5 per cent, and a vitamin supplement of Vi-Penta drops (La Roche) 0.5 ml. per kilo of diet. After this period, the diet was altered either by fasting or by changing its composition with respect to the percentage of protein, fat, or carbohydrate.

Observations were made on surviving liver slices prepared as for the Warburg technique. The animals were killed by a blow and the liver was immediately excised and sliced into 20 ml. of a bicarbonate buffered Krebs' solution at pH 7.2 containing phosphate (7). In control experiments, about 100 mg. of liver slices (dry weight) were transferred to a 250 ml. Erlenmeyer flask containing 20 ml. of the above solution, or, in test experiments, to a flask with the same solution containing 0.1 m sodium pyruvate and 0.04 m ammonium carbonate. The tissues were then shaken

^{*}Aided by a grant from the United States Public Health Service. A preliminary report has appeared (Federation Proc., 7, 17 (1948)).

in an atmosphere of 5 per cent CO₂ and 95 per cent O₂ for 2 hours at 37.5°. At the end of this period, they were removed by filtration, washed, dried at 104° overnight, and weighed. After having been made alkaline to phenolphthalein and boiled for the removal of ammonia (checked by nesslerization), the medium was analyzed for the increase in amino nitrogen by the nitrous acid method of Van Slyke (8).

Kritzmann (9) and Wiss (10) have demonstrated the formation of alanine from pyruvic acid and ammonia by liver slices and homogenate under experimental conditions essentially similar to ours. Experiments of our own with chromatography have shown that the increase in amino nitrogen is due almost entirely to formation of alanine. Traces of aspartic or glutamic acid are also formed. Since no amino nitrogen was found when pyruvate and ammonium carbonate were incubated in the medium without tissue, it was assumed that the small amount of amino nitrogen appearing in the flasks containing tissue and medium without substrates represented that which was simply leached from the tissue. This was deducted from the quantity found in the test flasks, and the difference represented the synthesized amino nitrogen. The results are given in terms of micrograms of amino nitrogen per mg. of dry weight of tissue per hour.

Results

The effect of fasting on the ability of the rat liver to synthesize alanine is shown in Fig. 1. The average amount of amino nitrogen formed by livers of twenty-two fed control animals is $7.1 \pm 0.26~\gamma$ per mg. of dry tissue per hour. Compared with this value, a profound depression in the formation of alanine following periods of fasting greater than 12 hours is observed. In ten experiments each, 24 and 48 hour fasts gave values of $1.2 \pm 0.15~\gamma$ per mg. and $1.1 \pm 0.14~\gamma$ per mg. respectively, while those for 72 and 96 hours were somewhat higher, but greatly below normal, being $2.6 \pm 0.65~$ and $2.7 \pm 1.04~$ respectively. In all cases, this deficiency of alanine synthesis produced by fasting could speedily be restored to normal by feeding the animals the standard diet. Thus, the synthesis amounted to $7.4~\gamma$ per mg. of tissue per hour when the standard diet was given for 24 hours after a 24 hour fast, and $6.2~\gamma$ per mg. when it was given after a 72 hour fast.

In order to ascertain whether this failure of synthesis after fasting was due to a deficiency of protein, carbohydrate, or fat, other groups of animals that had been placed on diets with varying amounts of these constituents were studied. Table I shows the results obtained when the animals had been fed these modified diets for 24 hours. The percentage composition in terms of total calories of each test diet, as well as the total

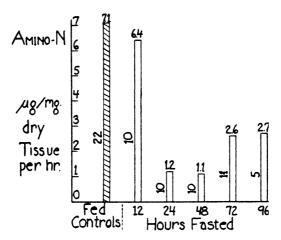


Fig. 1. Amino nitrogen synthesized by liver slices from rats after various periods of fasting. The numbers along the sides of the bars indicate the number of experiments, while those at the top of the bars are the average values obtained for each group of experiments in micrograms per mg.

TABLE I

Effect of Diet on Alanine Synthesis by Rat Liver

Amino nitrogen synthesized by liver slices from rats placed for 24 hours on the modified diets.

No. of	Com	position of	diet	Amount	Calories	Available	A	
experiments	Carbo- hydrate	Fat	Protein	caten	per 24 hrs.	carbohydrate	Amino nitrogen	
	per cent calories	per cent calories	per cent calories	gm. per 24 hrs.		gm. per 24 hrs.	γ per mg. dry tissue per hr.	
10	0	0	0	0	0	0	$1.2 \pm 0.15^{*}$	
1	0	100	0	13	117	1.3	1.8	
8	0	80	20	9	61	2.0	3.3 ± 0.69	
8	0	38	62	8	38	3.2	3.8 ± 0.41	
8	0	0	100	9.7	39	4.9	4.4 ± 0.59	
8	47	53	0	11	59	7.4	6.2 ± 0.35	
22	42 (Basic diet)	37	21	13	64	8.7	7.1 ± 0.26	
3	62	38	0	12	58	9.2	8.1 ± 1.02	
4	78	0	22	13	52	11.0	8.2 ± 0.33	

^{*} Deviation measure = standard deviation of the mean.

available carbohydrate, calculated as 10 per cent of the fat, 50 per cent of the protein, and 100 per cent of the carbohydrate eaten, is shown in Table I. In some cases, animals were kept on these diets for 48 and 72

hours, but, since the values obtained were not significantly different from those resulting after 24 hours, they were not included.

It appears from Table I that the deficiency in alanine synthesis by these livers is a function of the total available carbohydrate in the diet. Plotting the available carbohydrate against the micrograms of amino nitrogen produced by these tissues (Fig. 2) shows this relationship to be a linear one.

In two experiments, the addition of glucose to the medium in a concentration of 50 mg. per cent had no effect on the diminished synthesis of alanine by the liver from the fasted rat. An average value of 1.38 γ of alanine per mg. of dry tissue was obtained in these two experiments.

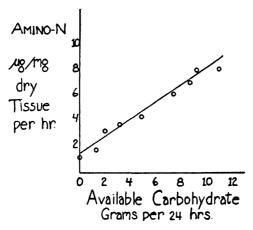


Fig. 2. Amino nitrogen synthesized by liver slices from rats placed for 24 hours on modified diets. Ordinate, micrograms of amino nitrogen per mg. of dry liver tissue per hour; abscissa, total amount of carbohydrate in gm. derivable from the diet eaten per 24 hours.

DISCUSSION

The most striking feature of these experiments is the rapidity and severity with which a dietary deficiency can affect a tissue function. Even in so short a period as 24 hours, fasting can almost completely abolish a synthetic process exhibited by the liver. The results indicate that the failure in alanine synthesis in these tissues is due to carbohydrate deficiency specifically, and not to a lack of non-specific sources of energy. This appears to be an indirect effect since addition of glucose in vitro did not increase the synthesis. A reductive amination would involve the need for a hydrogen donor, and it is not unreasonable to postulate that the deficiency, as observed in our experiments, was due to the lack of such a

donor. Although we confirm Wiss' (10) observation that the synthesis does not occur anaerobically, it is unlikely under our conditions of pyruvate excess that there should be a failure of carbohydrate oxidation, and hence a hydrogen donor deficiency below the pyruvate level. In searching for a possible place where such a deficiency might occur, we should take into account the observation of Kaplan and Greenberg (3) that a reduced content of adenosine triphosphate is found in the livers of carbohydrate-starved animals. This would favor a hydrogen donor deficiency at the triose stage of carbohydrate metabolism, and may explain the failure for the synthesis of alanine under the conditions of our experiments.

SUMMARY

Liver slices from fasted rats or from animals on diets deficient in carbohydrate show a marked reduction in the ability to synthesize alanine from pyruvic acid and ammonia.

The deficiency in alanine synthesis seems to be a direct function of the total available carbohydrate in the diet.

The addition of glucose to the medium does not increase the synthesis of alanine by the livers of fasted rats.

The possible mechanisms involved in this failure are discussed.

BIBLIOGRAPHY

- 1. Miller, L. L., J. Biol. Chem., 172, 113 (1948).
- 2. Potter, V. R., and Klug, H. L., Arch. Biochem., 12, 241 (1947).
- 3. Kaplan, N. O., and Greenberg, D. M., J. Biol. Chem., 156, 543 (1944).
- Seifter, S., Harkness, D. M., Feldman, B., Rubin, L., and Muntwyler, E., Federation Proc., 7, 17 (1948).
- 5. Kaplanskii, S., Berezovskaya, N., and Shmerling, Z., Biokhimiya, 10, 401 (1945).
- 6. Neber, M., Z. physiol. Chem., 234, 83 (1935).
- 7. Krebs, H. A., and Henseleit, K., Z. physiol. Chem., 210, 33 (1932).
- 8. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore (1932).
- 9. Kritzmann, M. G., J. Biol. Chem., 167, 77 (1947).
- 10. Wiss, O., Helv. chim. acta, 31, 1189 (1948).

THE SPECTROPHOTOMETRIC DETERMINATION OF CARBON MONOXIDE

By NIELS C. KLENDSHOJ, MILTON FELDSTEIN, AND ALICE L. SPRAGUE (From the University of Buffalo School of Medicine and the Buffalo General Hospital, Buffalo)

(Received for publication, October 29, 1949)

The spectrophotometry of carboxyhemoglobin and oxyhemoglobin has been studied by Cherbuliez (1), Hüfner (2), Newcomer (3), Haurowitz (4), Kennedy (5), and Warburg (6). The absorption curves of these pigments (taken from a compilation of their results) are shown in Fig. 1.

In 1900 Hüfner (7) worked out a spectrophotometric method for the determination of carboxyhemoglobin in the presence of oxyhemoglobin. He utilized the principle that, in mixtures of two pigments, if the quotient D_1/D_{2^*} (in which D_1 equals the optical density at wave-length I and D_2 equals the optical density at wave-length II) of one pigment is A, and the quotient D_1/D_2 for the other pigment is B, then any mixture of the two pigments will have a D_1/D_2 value somewhere between A and B, depending on the concentration of the pigments. His mathematical treatment is similar to that presented by Gibb (8).

Hüfner chose the wave-lengths 541 and 560 m μ and showed that D_{541}/D_{560} for pure carboxyhemoglobin is 1.10 and for pure oxyhemoglobin 1.58, and that mixtures of the two have D_1/D_2 values between these two figures. Heilmeyer (9) substituted the quotient D_{576}/D_{560} which is 0.88 for carboxyhemoglobin and is 1.72 for oxyhemoglobin. Mixtures of the two have values between these two limits. The difference between the quotient for pure carboxyhemoglobin and the quotient for pure oxyhemoglobin in this case is more substantial than the difference obtained with Hüfner's quotient. With both of these quotients, however, the difference in the values obtained with pure pigments is still quite small and requires extremely accurate technique and instruments.

We felt that a more satisfactory quotient could be obtained by measurement of the optical density of carboxyhemoglobin in the presence of reduced hemoglobin. Oxyhemoglobin is completely reduced in the presence of small amounts of sodium hydrosulfite, whereas carboxyhemoglobin is not affected. The curves for reduced oxyhemoglobin and "reduced" carboxyhemoglobin are shown in Fig. 2. From a consideration of these curves, the wave-lengths 555 and 480 m μ have been chosen as most suitable for the determination of carboxyhemoglobin in the mixture.

Procedure

1 ml. of oxalated blood is diluted to 100 ml. with 0.4 per cent ammonia. 3 ml. are placed in a cuvette and about 10 mg. of pure sodium hydrosulfite

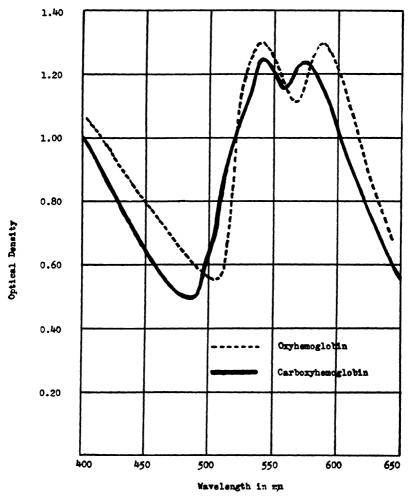


Fig. 1. Absorption curves of oxyhemoglobin and carboxyhemoglobin (1:100 dilution in 0.4 per cent ammonia).

are added. The solution is mixed by inverting ten times and the densities at 555 and 480 m μ are read at once against 0.4 per cent ammonia as a blank. The value of D_{556}/D_{480} is calculated and the percentage of carboxyhemoglobin is read from the prepared concentration-quotient curve. All read-

ings were made on the Beckman model DU quartz spectrophotometer in 1 cm. quartz cells.

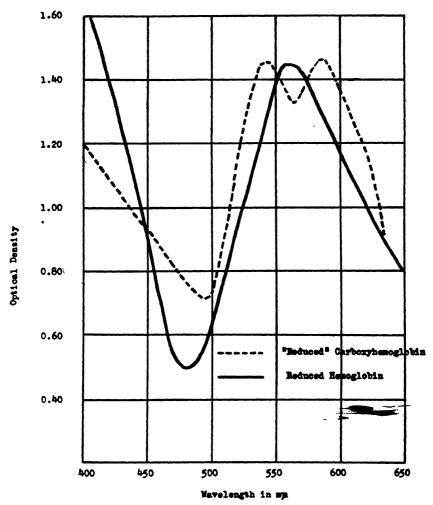


Fig. 2. Absorption curves of reduced hemoglobin and "reduced" carboxyhemoglobin (1:100 dilution in 0.4 per cent ammonia).

Determination of Quotient D₅₅₅/D₄₅₀ for Carboxyhemoglobin and Reduced Hemoglobin—Twenty samples of oxalated blood from hospital patients known not to have been exposed to carbon monoxide were obtained. 5 ml. of the sample were placed in a 250 ml. separatory funnel, and the air remaining was displaced with pure oxygen. The funnel was capped and

rotated for $\frac{1}{2}$ hour to saturate the hemoglobin with oxygen. A second 5 ml. sample of the same blood was treated similarly, with carbon monoxide to displace the air in the separatory funnel. In this manner, samples of oxyhemoglobin and carboxyhemoglobin were prepared. 1 ml. portions of each were diluted to 100 ml. with 0.4 per cent ammonia and analyzed as outlined in the "Procedure." The quotient D_{555}/D_{430} for reduced oxyhemoglobin is 3.15 ± 0.05 , and for "reduced" carboxyhemoglobin 1.94 ± 0.05 . These figures agree closely with quotients calculated from data presented by Heilmeyer (10).

Preparation of Concentration-Quotient Curve—Mixtures of blood containing varying amounts of carbon monoxide were prepared and analyzed for carbon monoxide by the Van Slyke volumetric method (11). These samples were then diluted 1:100 with 0.4 per cent ammonia and analyzed ac-

 ${\bf TABLE~I} \\ {\bf Relationship~between~Per~Cent~Carbon~Monoxide~and~Quotient~D_{555}/D_{450}~for~Reduced} \\ {\bf Hemoglobin~and~``Reduced''~Carboxyhemoglobin} \\$

Per cent carbon monoxide by Van Slyke analysis	Quotient Dass/Daso
0	3.15
10	2.97
20	2.81
30	2.67
40	2.54
50	2.41
60	2.31
80	2.12
100	1.94

cording to the procedure outlined above. The results are shown in Table I. The concentration-quotient curve is shown in Fig. 3.

As a final check on the accuracy of the method, a series of bloods taken from patients exposed to carbon monoxide was analyzed by both the Van Slyke method and the spectrophotometric method, by the use of Heilmeyer's quotient, Hüfner's quotient, and the reduced quotient D_{555}/D_{480} . These results are shown in Table II.

From a consideration of these figures, it can be seen that for values of carbon monoxide saturation below 20 per cent the three spectrophotometric methods give results closely comparable with those by the Van Slyke gasometric method. However, with values above 20 per cent, and especially in the range 50 to 80 per cent, only the reduced quotient D_{555}/D_{450} gives results which agree closely with the Van Slyke procedure.

The presence of other pigments introduces errors which invalidate the

two pigment calculation (8). However, methemoglobin does not interfere since it is reduced with sodium hydrosulfite ((10) p. 105). That methemoglobin does not interfere was shown by analysis of several blood samples

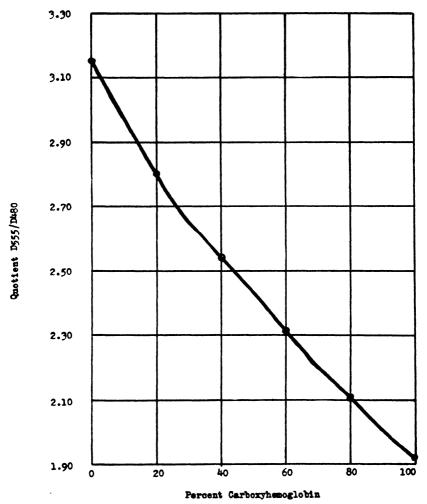


Fig. 3. Concentration-quotient curve. Proportion of carboxyhemoglobin in a mixture of oxyhemoglobin and carboxyhemoglobin determined by means of the reduced quotient $D_{\text{MS}}/D_{\text{des}}$.

containing both methemoglobin and carboxyhemoglobin. These results are shown in Table III. The percentage of methemoglobin was determined by Heilmeyer's method ((10) pp. 103-104).

Table II

Comparison of Results for Carbon Monoxide Content of Blood by Various Methods

	Per cent carbon monoxide saturation						
Case No.	Van Slyke gasometric method	Hüfner's method, D_{641}/D_{660}	Heilmeyer's method,	Reduced method			
1	74.1	55.2	51.4	73.2			
2	82.0	68.0	64.0	81.5			
3	73.5	44.0	43.0	72.5			
4	18.7	13.0	19.0	19.0			
5	77.8	59.0	54.0	78.5			
6	10.7	9.5	9.5	10.0			
7	14.5	14.5	13.9	14.0			
8	83.0	69.0	72.0	82.0			
9	39.6	27.0	30.0	40.0			
10	71.5	60.0	55.0	72.0			
11	87.0	70.0	67.0	86.0			
12	31.5	23.0	22.0	30.0			
13	43.5	37.0	33.0	41.0			
14	29.0	20.0	19.0	30.0			

Table III

Determination of Carbon Monoxide in Presence of Methemoglobin

Per cent methemoglobin	Per cent carbon monoxide			
rer cent methemogroun	Van Slyke method	Quotient Dass/Dave		
7.5	0	0		
17.2	1.5	2.0		
9.3	10.9	9.9		
72	0	0		
9.0	2.5	3.2		
26	0	0		
16	31	32		
47	0	0		

Table IV

Stability of Reduced Quotient D₅₅₅/D₄₈₀ of Unhamolyzed Blood in Determination of Carbon Monoxide

Time on ice	Per cent carbon monoxide				
Time on ice	Van Slyke method	Quotient method, Duss/Daso			
hrs.					
0	82.0	81.0			
4	82.2	81.0			
8	82.0	81.5			
16	82.4	81.0			
24	82.2	81.4			

Blood which is hemolyzed, or which has been standing for several days, as is the usual case with post mortem samples, contains other pigments arising from the breakdown of hemoglobin which make it unsuitable for analysis by this method. If precautions are taken to avoid hemolysis, the reduced quotient of refrigerated blood remains constant for several days. This is shown in Table IV.

SUMMARY

A method for the spectrophotometric determination of carbon monoxide in blood has been presented. The method utilizes the quotient D_{555}/D_{490} in the two pigment solution consisting of reduced hemoglobin and carboxyhemoglobin. Oxalated blood is diluted 1:100 with 0.4 per cent ammonia, reduced with sodium hydrosulfite, and the ratio D_{555}/D_{490} is determined. The per cent saturation of carbon monoxide is read from a prepared graph.

BIBLIOGRAPHY

- 1. Chefbuliez, E., Études spectrophotométriques du sang oxicarboné, Paris (1890).
- 2. Hüfner, G., Arch. Physiol., 130 (1894).
- 3. Newcomer, H. S., J. Biol. Chem., 37, 465 (1919).
- 4. Haurowitz, F., Novin Lekarsk W. Poznanin. Festschrift Wacholz, 83, 10 (1926).
- 5. Kennedy, R. P., Am. J. Physiol., 79, 346 (1927).
- 6. Warburg, O., Biochem. Z., 214, 38 (1929).
- 7. Hüfner, G., Arch. Anat. u. Physiol., Physiol. Abt., 39, 63 (1900).
- 8. Gibb, T. R. P., Optical methods of chemical analysis, New York, 113 (1942).
- 9. Heilmeyer, L., Biochem. Z., 223, 352 (1930).
- 10. Heilmeyer, L., Spectrophotometry in medicine, London, pt. 2, 65-94 (1943).
- 11. Van Slyke, D. D., and Salvesen, H. A., J. Biol. Chem., 40, 103 (1919).

MEASUREMENT OF SPECIFIC GRAVITIES OF WHOLE BLOOD AND PLASMA BY STANDARD COPPER SULFATE SOLUTIONS*

By ROBERT A. PHILLIPS,**† DONALD D. VAN SLYKE,† PAUL B. HAMILTON,§ VINCENT P. DOLE,|| KENDALL EMERSON, Jr.,||¶

AND REGINALD M. ARCHIBALD

WITH THE TECHNICAL ASSISTANCE OF EMILY G. STANLEY

(From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, July 18, 1949)

The specific gravity measurements are carried out by letting drops of plasma or whole blood fall into a graded series of copper sulfate solutions of known specific gravities, and noting whether the drops rise or fall in the solutions. Each drop, on entering a solution, becomes encased in a sac of copper proteinate and remains discrete, without change of gravity, for 10 or 15 seconds, during which its rise or fall reveals the relation of its gravity to that of the solution. The drops do not have to be of definite size; hence no special pipette is needed for delivering them. No temperature correction is needed, because the temperature coefficients of expansion of the copper sulfate solutions approximate closely those of blood and plasma; regardless of the temperature at which the analysis is made, gravities are given in terms of D_{25}^{25} . The copper sulfate solution automatically cleans itself after each test, because within a minute or two the material of the drop settles to the bottom as a precipitate.

The principle of adding drops of blood to a series of liquids of graded densities and noting whether the drops rise or fall has long been applied, but has hitherto suffered certain limitations. Roy (3) in 1884 used standard aqueous salt solutions to measure the gravity of whole blood, and similar standards were employed for blood by Rogers (4) in his classic studies of dehydration in cholera. The gravity of whole blood can be esti-

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

Preliminary publications of the method appeared in the medical journals of the Armed Services (1, 2).

- ** Commander, Medical Corps, United States Navy.
- † Present address, United States Naval Medical Research Unit No. 3, care of the American Embassy, Cairo, Egypt.
 - ‡ Present address, Brookhaven National Laboratory, Upton, New York.
 - § Present address, Alfred I. du Pont Institute, Wilmington, Delaware.
 - || Lieutenant Commander, Medical Corps, United States Naval Reserve.
 - ¶ Present address, Peter Bent Brigham Hospital, Boston, Massachusetts.

mated fairly well with standard aqueous solutions of salt or glycerol, but drops of plasma disperse so rapidly in such solutions that accurate observation is difficult. To overcome this difficulty, standards composed of water-immiscible liquids, such as benzene and chloroform, have been used, but such liquids have temperature coefficients that are several times that of plasma or blood, and hence cannot be employed for precise results without accurate temperature control. Furthermore, the organic mixtures that have been generally used are subject to change from different evaporation rates of the components.

The above disadvantages of graded density standards have, in the present method, been overcome by the use of aqueous standard solutions that coagulate proteins, and that have almost exactly the same temperature coefficients of expansion as blood or plasma of corresponding densities. The copper sulfate can be replaced by other protein-coagulating solutes, such as zinc sulfate, or sodium chloride plus picric acid.

For measurement of blood and plasma gravities with drop samples, two accurate and elegant methods have been available, the falling drop method of Barbour and Hamilton (5) and the gradient tube method of Jacobsen and Linderstrøm-Lang (6) as applied by Lowry and Hastings (7) and Lowry and Hunter (8) Each of these methods has the advantage over the copper sulfate method of requiring only a single drop of blood or plasma, instead of 3 or 4 drops. The copper sulfate procedure has, however, certain advantages: a stable base is not required (accurate results can be obtained on shipboard), calibration is unnecessary, the apparatus (set of standards) can be supplied ready for operation from central laboratories and can be used without modification of technique, restandardization, or use of temperature corrections, at temperatures from 4-40°. Drops of constant, accurately measured size, such as are required by the Barbour-Hamilton method, are not needed for the copper sulfate procedure, nor is daily restandardization such as is required by the gradient tube.

A stock copper sulfate solution is first prepared which has at 25° a density 1.1000 times that of water at that temperature. From this stock solution the series of standard solutions is prepared by dilution.

PREPARATION OF STOCK SOLUTION OF $D_{25}^{25} = 1.1000$

The stock solution contains, at 25°, 159.0 gm. of CuSO₄·5H₂O per liter. At any temperature it contains 5.897 gm. of solvent water per 1 gm. of CuSO₄·5H₂O.

For preparation of solutions by weight, the sulfate must have the exact composition CuSO₄·5H₂O, with theoretical H₂O content. The H₂O is determined by heating 2 or 3 gm. of the sulfate in open Pyrex weighing bottles at 300-350° until the weight is constant, which requires 2 or 3

hours. The loss in weight should be 36.06 ± 0.2 per cent. Crystals of U. S. P. sulfate do not usually meet this requirement. "Analytical reagent" grade usually has the correct composition.

The $CuSO_4 \cdot 5H_2O$ and water are mixed in the ratio of 170 gm. of crystals to 1002.5 gm. of water. It is convenient to use sealed bottles containing 170 ± 0.1 gm. of "fine crystals" of analyzed purity.

Table I

Volumes of Water to Add to 170.0 Gm. of $CuSO_4 \cdot 5H_2O$ to Prepare Stock Solution of $D_{\cdot \cdot \cdot}^{10} = 1.1000^{\circ}$

Temperature of water	Ml. of water to 170 gm. CuSO ₄ ·5H ₂ O
℃ .	
10	1003.6
12	1003.8
14	1004.0
16	1004.3
18	1004.7
20	1005.1
22	1005.5
24	1006.0
26	1006.5
28	1007.0
30	1007.7
32	1008.3
34	1008.9
36	1009.6
38	1010.4
40	1011.2

^{*} The solution contains 1002.5 gm. of water per 170 gm. of CuSO₄·5H₂. The volumes of water given in the table are 0.8 ml. more than theoretical in order to allow for adherence of this amount to the inside of the flask after 2 minutes drainage.

To make 3.2 liters of stock solution, sufficient for a set of 100 ml. standards, transfer three of the 170 gm. portions of sulfate quantitatively to a 4 liter bottle. Fill a 1 liter volumetric flask to the mark with distilled water. Take the temperature of the water. Then add from a burette

¹ Bottles containing 170.0 gm. of weighed "fine crystals" can be obtained from E. H. Sargent and Company, 155-165 East Superior Street, Chicago 11, Illinois. The CuSO₄·5H₂O sold as "fine crystals," smaller than wheat grains, is preferable to that in large crystals, because of the greater ease with which the smaller crystals dissolve.

enough additional water to the liter in the flask to bring the volume up to that indicated in Table I for addition to 170 gm. of sulfate. Empty the water from the flask into the bottle containing the sulfate. Let the water from the upturned flask drain into the bottle 2 minutes. In this manner measure into the bottle one portion of water from the 1 liter flask for each 170 gm. portion of copper sulfate.

Particularly if there is any doubt concerning the water content of the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ crystals, it is desirable to check the specific gravity of the solution by a pycnometer with the technique described in the experimental part. If the D_{25}^{25} obtained differs by more than 0.0001 from 1.1000, it is adjusted by adding, per liter of solution, 1 ml. of water for each 0.0001 by which the D_{25}^{25} is too high, or approximately 1 ml. of saturated copper sulfate solution for each 0.0001 by which the D_{25}^{25} is too low.²

PREPARATION OF STANDARD COPPER SULFATE SOLUTIONS BY DILUTION OF STOCK SOLUTION

The procedure will be described for making 100 ml. portions of the standard solutions with gravities 0.001 unit apart. To receive the solutions 100 ml. cylindrical prescription bottles with screw caps are cheap and satisfactory, 64 being required for the set. Each bottle is labeled on the side and also marked on the cap with the gravity of the solution that it is to contain, indicated in Table II as D_{25}^{25} . To measure the stock solution it is convenient to have a 25 ml. burette and a 25 ml. pipette attached to the bottle of stock solution by filling devices. A single 100 ml. volumetric flask completes the required apparatus; its mark should not be more than 25 mm. above the level where the neck widens into the bulb.

To prepare each standard the volume of stock solution indicated in Table II is measured into the 100 ml. flask, the volume up to 25 or 50 ml. being measured by pipette, the rest by burette. After the solution is in the flask, water is added in a rapid stream until the surface approaches the neck of the flask. During this addition the water is mixed with the solution by rotating the flask. Then the addition of water is continued, without rotation, until the 100 ml. mark is reached, and the flask is inverted to complete mixing. The preliminary mixing attained before the neck of the

² The density symbol D_{15}^{25} or D_{t}^{t} used in this paper indicates the ratio (weight of solution)/(weight of water), both weights being those of the same volume of fluid at the same temperature when weighed under the same conditions, without correction of either weight for the buoyant effect of air, which would be approximately 1.06 mg. per ml. of fluid. The uncorrected D_{t}^{t} unit is used because it is common in biological work. As is seen from Table IV, the effect of correction for the buoyant effect of air on D_{t}^{t} values within the range of blood and plasma is significant only in the fifth decimal place.

PHILLIPS, VAN SLYKE, HAMILTON, DOLE, EMERSON, AND ARCHIBALD

Table II

Volumes of Stock CuSO₄ Solution of $D_{ss}^{u} = 1.1000$ to Dilute to 100 Ml. to Make Standard Solutions for Plasma and Blood

Standard solution	ons for plasma*		Standard solution	s for whole blood*	
Gravity of plasma balanced by standard	Volume of stock solution per 100 ml. standard	Gravity of blood balanced by standard	Volume of stock solution per 100 ml. standard	Gravity of blood balanced by standard	Volume of stock solution per 100 ml. standard
D 25	ml.	D 16	ml.	D 26	ml.
1.015	13.9	1.035	34.3	1.055	54.3
16	14.9	36	35.3	56	55.3
17	15.85	37	36.3	57	56.3
18	16.8	38	37.25	58	57.3
19	17.8	39	38.2	59	58.3
20	18.8	40	3 9.2	60	59.3
21	19.8	41	40.2	61	60.3
22	20.75	42	41.2	62	61.3
23	21.7	43	42.2	63	62.3
24	22.7	44	43.2	64	63.35
25	23.7	45	44.2	65	64.4
26	24.7	46	45.2	66	65.4
27	25.7	47	46.2	67	66.4
28	26.65	48	47.2	68	67.4
29	27.6	49	48.2	69	68.4
30	28.6	50	49.2	70	69.4
31	29.6	51	50.2	71	70.4
32	30.6	52	51.25	72	71.45
33	31.6	53	52.25	73	72.5
34	32.6	54	53.3	74	73.5
35	33.6	55	54.3	75	74.5
36	34.6				

^{*} For reasons discussed under "Preparation of standard copper sulfate solutions by dilution of stock solution," the standard solutions for plasma are made lighter by 0.0006 gravity unit than the drops of plasma that they balance, and the standard solutions for whole blood are made 0.0001 unit heavier than the drops of whole blood that they balance.

flask is reached is sufficient to prevent measurable shrinkage in the final mixing. The solution is decanted into a labeled bottle.

After the transfer of each standard, the 100 ml. flask is rinsed with water, and is used to prepare the next standard of the series.

For holding the bottles of standards during use a convenient rack can be made from a box about 35 by 60 by 6 cm., with thin crosspieces dividing it into forty-five sections to hold the standards that cover the range ordinarily used, viz., gravities 1.018 to 1.062. At the middle of each end is attached an upright board 6 by 40 cm. The two boards are connected at the top by another which serves as a handle to carry the set, and also serves as a platform on which to place bottles near the level of the eye when they are used for gravity determinations.

When analyses are not numerous, heavy walled test-tubes of not less than 15 ml. inner diameter, filled to within 3 cm. of the top, and closed with rubber stoppers, may be more convenient than bottles as containers of the standard solutions during the analyses.

Provided both stock solution and water are at the *same* temperature when they are mixed according to Table II, the weight ratio of solvent water to copper sulfate is not significantly affected by temperature variations between 10° and 40°. Because the coefficient of expansion of the stock solution is greater than that of water, there is a slight effect, but it is such that a mixture of stock solution and water by volume that would have, when prepared at 25°, a D_{25}^{25} of 1.050, would have, if prepared with the same volumes measured at 10°, a D_{25}^{25} of 1.05005.

The standard solutions prepared according to Table II have slightly different gravities from the D_{25}^{25} values of plasma and blood indicated in Table II as "balanced by" the standards. As shown in the experimental part (Table VI), plasma drops balance (neither rise nor fall) in copper sulfate solutions that average lighter than the plasma by 0.0007 gravity unit. Hence, to indicate the correct plasma gravities, the standard solutions for plasma are prepared by Table II to have D_{25}^{25} values 0.0007 \pm 0.0001 lighter than the values shown in the first column of the table. Each standard is prepared to have, before it is used, a gravity lower by 0.0006 unit than it is labeled, and during use the gravity falls 0.0002 unit more before the standard is discarded (see "Renewal of standard solutions") so that the D_{25}^{25} range of each standard during the period of its use is 0.0007 \pm 0.0001 lower than it is labeled. A plasma of gravity 1.0270 balances in a copper sulfate standard solution marked 1.0270, but the actual gravity of the standard is 1.0263.

Drops of whole blood balance in copper sulfate solutions that on the average have the same gravity as the blood (Table VII). To allow for a decrease of 0.0002 in the gravity of standards for whole blood during use, the standards are made heavier by 0.0001 unit than the D_{25}^{25} values indicated in Table II.³

* The dilution table given for preparation of copper sulfate solutions in the preliminary reports of the copper sulfate method (1, 2) gave for both plasma and blood

DRAWING AND PREPARATION OF BLOOD

For samples permitting measurement of the gravity of plasma or serum as well as that of whole blood, procedures are described for venous blood in portions of 1 to 5 ml., and for capillary blood in portions of 0.5 ml. For the gravity of whole blood alone, a procedure for use with finger blood portions of about 0.1 ml. is detailed.

Venous Blood, Portions of 1 to 5 Ml.

Preparation of Oxalated or Heparinized Test-Tubes or Centrifuge Tubes— Test-tubes of heavy walled Pyrex glass or of plastic, of capacity equal to about twice the volume of blood used, are prepared in advance by pipetting into each tube 0.05 ml. of either a 0.4 per cent solution of heparin or a 2 per cent solution of Heller and Paul's (11) oxalate mixture (12 gm. of ammonium oxalate and 8 gm. of potassium oxalate to 1 liter), for each ml. of blood that the tube is to receive. The solution is spread in a film over the lower half of the tube, and is dried in an incubator, or in air warmed to not over 60°, or in a vacuum desiccator. Each tube, thus charged with anticoagulant, is provided on the outside with a mark to indicate the volume of blood that it is to receive. It is essential to maintain the above proportion of anticoagulant to blood volume; viz., 1 mg. of oxalate or 0.2 mg. of heparin per ml. of blood. If less anticoagulant is used, coagulation may occur. If more anticoagulant in the form of oxalate is used, it will increase significantly the gravity of the blood and plasma (see Table VIII). Unmeasured proportions of oxalate must not be used, or gross errors may result in the gravities of blood and plasma, and in hemoglobin and plasma protein concentrations calculated from the gravities.

standards 0.0001 to 0.0002 gravity unit lighter than is indicated in these reports, instead of 0.0007 lighter for plasma and identical for blood as in the present paper. Comparison of plasma and blood gravities determined by pycnometer with the gravities of the copper sulfate solutions in which the drops balance (Tables VI and VII) has led to changing the preparation of the standards (Table II) so that they will indicate without correction the blood and plasma gravities determined by pycnometer. The use of the former standards introduced no inaccuracy into the calculation of plasma protein and blood hemoglobin concentrations, because the formulas for the calculations were based on comparisons of protein and Hb concentrations with gravities estimated by the same copper sulfate standards. Laboratories using the former standards, and the formulas and nomograms developed for calculations of plasma proteins (1, b) and blood Hb from gravities obtained with them, can continue to do so without error. If the present standards are used, however, the formulas and nomogram developed in the succeeding papers (9, 10) are to be employed. Use of the former formulas with gravities determined by the present standards would cause errors of about +0.2 gm. of calculated plasma protein per 100 ml, of plasma and -0.2 gm. of Hb per 100 ml, of blood.

With regard to choice between heparin and oxalate, heparin is preferable if the gravity measurements are to be made within a few hours, because heparin does not measurably affect the gravity. If heparinized blood stands till the next day, however, small clots may form that interfere with the gravity measurement. Also, in some specimens of heparinized blood that have stood more than 18 hours in the refrigerator, some change has been seen to occur as a result of which drops of the blood, when let fall into copper sulfate solution, fail to break through the surface film of the solution, but remain attached to the film by streamers which prevent observation of the gravity. This phenomenon has not been observed in oxalated blood, nor in freshly heparinized blood.

Drawing Venous Blood—Tourniquets should not be applied longer than 1 minute. Longer applications may force so much fluid out of the blood that concentrations of both plasma protein and blood hemoglobin are measurably increased.

Handling Venous Blood; without Anticoagulant—If the specific gravity of whole blood only, or of whole blood and serum, is to be measured, the blood is drawn into a syringe, and drops are at once delivered from the needle into the copper sulfate solutions to determine whole blood gravity. Then the remainder of the blood may be transferred to a centrifuge tube and allowed to clot in order to obtain serum.

With Anticoagulant—If results for whole blood and plasma are desired, a measured amount of anticoagulant is added, as described above, to the tube that is to receive the blood. The gravity of the whole blood may be determined either immediately after drawing, by dropping blood from the syringe needle into copper sulfate standards, or after transfer to the oxalated or heparinized tube, before centrifugation. In the latter case it is necessary to mix the cells and plasma thoroughly before the blood sample is withdrawn from the tube into a dropper or syringe. To attain complete mixing either invert the tube containing the oxalated or heparinized blood ten times, or stir with a glass rod with a mushroom end, which is raised and lowered through the blood ten times, just before the sample is drawn into the dropper or syringe. Gross error in the estimation of blood gravity and in gravity-calculated hemoglobin could result if the blood sample were taken from blood in which partial settling of the cells had occurred.

Capillary Blood, 0.5 Portions for Gravity of Both Whole Blood and Plasma4

A small test-tube is prepared, as shown in Fig. 1, with a rubber cap through which pass two short capillary tubes of about 1 mm. bore. The

⁴ Procedure devised by L. E. Farr (personal communication). The rubber caps are made from stoppers catalogued as No. 2319-B by the Arthur H. Thomas Com-

longer capillary serves to admit blood, the other to let out the replaced air. The inlet capillary should be cleaned with chromic acid before it is placed in the rubber cap; otherwise blood may not readily flow through it. The test-tube is marked at the level indicating 0.5 ml., and is coated within by a dried film from 0.025 ml. of the heparin or oxalate solution described above for venous blood.

A doep cut is made in a finger, or on the back of a thumb just below the nail, and the exuding blood is drawn by capillary attraction into the long inlet capillary, while the test-tube is held at sufficient downward slant to cause the blood to drop down into the tube. As soon as 0.5 ml. of blood has been collected, it is well stirred with a small rod, and a portion is with-



Fig. 1. Farr tube, 6 by 50 mm. outside measurement, arranged to receive 0.5 ml. of capillary blood, for specific gravity of both blood and plasma.

drawn by a small syringe or medicine dropper, and used to determine the whole blood gravity.

The remainder of the blood is centrifuged, and the supernatant plasma similarly withdrawn for gravity estimation.

Capillary Blood, 4 or 5 Drops, for Gravity of Whole Blood

Glass capillary tubes, of 1 to 1.5 mm. bore and about 150 mm. in length, are used.⁵ It is convenient, for expulsion of blood drops, to provide the

pany for serum bottles. The portion of stopper that is for insertion into a bottle is cut off to form the cap shown in Fig. 1. The cap is pierced with a needle to provide holes to receive the capillary tubes. The test-tubes are fermentation tubes, and are catalogued by Thomas as No. 9446, thick wall, without lip, 50 by 6 mm. outside dimensions.

⁵ Obtainable from the Kimble Glass Company, Vineland, New Jersey. Capillaries sold for melting point tubes may also be used.

tubes with small rubber bulbs, shown in Fig. 2, open at both ends, used with "vaccine tubes." The bores of the glass capillaries must be clean in order to draw in blood by capillary attraction. The tubes can be cleaned by soaking them in chromic-sulfuric acid cleaning mixture in a test-tube, then rinsing twenty times with distilled water, and drying in an oven.

The rubber bulbs shown in Fig. 2 are convenient, but not necessary. Without them one can expel the greater part of the blood from a filled capillary by raising it upright, and the last drop can be expelled by gentle pressure of the breath.

A finger or ear lobe is cleaned with 70 per cent alcohol, wiped perfectly dry with sterile gauze or cotton, and pricked with a lancet. The cut must

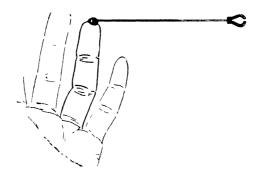


Fig. 2. Drawing capillary blood from finger for specific gravity of whole blood

be deep enough to yield a large drop of blood. The size of the drop may be increased by massaging the finger from the base towards the tip. One end of a glass capillary is immersed in the drop while the capillary is held horizontally. If the bore is clean, the first half will fill without tilting the tube downwards at all. To fill the last half it is necessary to slant the tube slightly downwards from the finger (Fig. 2), but as little slant as possible is used or a bubble may be drawn in.

By vaselining slightly the delivery end before the drops are delivered, the size of the drops may be diminished so that 1 or 2 more drops from the tubeful are obtained. The blood is immediately used for the gravity test, before there is time for clotting to begin. It serves for approximate hemoglobin estimations (10).

⁶ The bulbs are obtainable from the West Rubber Company, 117 Shackamaxon Street, Philadelphia, Pennsylvania.

SPECIFIC GRAVITY DETERMINATION

Procedure

The drop of serum, plasma, or whole blood is delivered from a height of about 1 cm. above the solution⁷ from a medicine dropper, a capillary, or a



Fig. 3. Dropping blood into a standard copper sulfate solution

syringe needle. It is preferable to use small drops for the reason that they permit more tests before the standard solution must be changed. There-

⁷ If the drop strikes with too little force, it may not break through the surface film. On the other hand, if the drop falls from too great a height, it may be broken up on striking the solution, or its momentum may carry it too far below the surface; the momentum should be only enough to carry a drop, of gravity slightly less than that of

fore a syringe needle serves well, and a medicine dropper with a fine tip is preferable to one with a coarse tip.⁸ Greasing the outside of the tip with vaseline also reduces the size of the drop. When the drop is delivered, it is convenient to steady the syringe or dropper on the edge of the bottle (Fig. 3).

The delivered drop breaks through the surface film of the solution and its momentum carries it 1 or 2 cm. below the surface; within 5 seconds the momentum of the fall is lost, and the drop then either begins to rise, becomes stationary, or continues to fall. The gravity of the drop relative to that of the solution does not change appreciably until the drop has been immersed in the solution for another 10 or 15 seconds, and there is ample time to note its behavior during this interval. If the drop is lighter than the test solution, it will rise, perhaps only a few mm., and may begin to sink immediately afterward. If the drop is of a gravity to balance in the standard test solution, it will become stationary for a few seconds and then fall. If the drop is heavier, it will continue to fall during the entire interval. In summary, the behavior during the 10 seconds after the drop has lost the momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution; if it rises at all during this period, it is lighter than the standard.

The following order of using the solutions with plasma is usually followed. Place the first drop in the 1.027 standard, with the gravity of average normal plasma. According to whether the drop sinks or rises, place the second drop four points away, at 1.031 or 1.023. If the plasma gravity is within the range 1.023 to 1.031, test intermediate points between the first two as in the example given below. If the plasma gravity is outside the range 1.023 to 1.031, make the second test at 1.019 or 1.035.

A similar order is followed with whole blood, the first test being made with the standard of gravity 1.056 if the blood is a woman's, 1.059 if a man's. If it is known in advance that the subject is low in plasma proteins or hemoglobin, the initial test may be made with a standard of lower gravity; e.g., 1.023 for plasma, 1.050 for blood.

Example—The following example shows how, by bracketing on the probable extremes of a plasma's gravity range and then testing intermediate points, one can with 4 drops estimate the specific gravity with a precision that can be duplicated within 0.0002 unit.

the solution, 1 or 2 cm. below the surface. A fall of about 1 cm. usually gives the right striking force. If room temperature is above 30°, a fall of less than 1 cm. suffices, since both the viscosity of the solution and the strength of the surface film are less than at lower temperatures.

⁸ When a medicine dropper is used, it is convenient to apply a screw clamp to the rubber bulb. By manipulating the screw one can easily draw in a sample of desired volume, and expel a series of drops without forming bubbles by admitting air into the tip after expulsion of any drop.

4 successive drops gave the following results, in which the figures indicate the gravities of the standards, and plus or minus after each gravity figure indicates that the plasma gravity was greater or less than that indicated by the preceding figure: 1.027 +, 1.031 -, 1.029 +, 1.030 -. The plasma gravity was greater than 1.029 and less than 1.030, and could therefore be placed at 1.0295, within less than ± 0.0005 .

By noting the relative rate of fall or rise in the two adjacent solutions, 1.029 and 1.030, it was further obvious that the plasma was nearer 1.029 than 1.030. Being less than 1.0295 and greater than 1.0290, it could be placed at 1.0293, within ± 0.0002 .

Special Points and Precautions

1. Surface Film Effects in Analyses—Occasionally a drop will fail to make a clean break through the surface of the copper sulfate solution, and remain attached by a tentacle to the film. In this case the drop is lifted from the surface by a rod or stick, or is made to sink by tapping the tube, and a fresh drop is tried.

After each test, one makes sure that the surface film is left clean and free from fragments. If any are left on the film, they are likely to prevent a clean break through of the drop in the next test. Fragments caught in the surface film can usually be detached by tapping the tube; they then sink to the bottom. Sometimes, however, a fragment of fatty nature or holding a bubble will continue to float on the surface. Such fragments are removed with a rod or stick.

- 2. Avoidance of Bubbles—Even a minute air bubble attached to a drop of blood or plasma will make it float. While drawing finger blood into a capillary tube (Fig. 2), make sure that the tip is kept immersed, so that no air bubbles will be drawn in, and hold the tube horizontal or at only a slight downward slant from the finger. When a drop is expelled into copper sulfate solution, enough excess blood or plasma should be present to leave some in the dropper. After releasing a drop from a medicine dropper without a screw clamp, one is likely to draw into the tip a bubble, which may attach itself to the next drop; the use of a syringe, or of a medicine dropper provided with a screw clamp, eliminates this source of error.
- 3. Temperature—The temperature may vary from 4-40° without significantly affecting results, so long as the copper sulfate solutions and the blood or plasma are within 5° of the same temperature.

The change of gravity per 1° change in temperature is about 0.0003. However, the error caused by initial difference in temperature between the blood or plasma and the standard copper sulfate solution is much less, because when a drop of blood or plasma enters the copper sulfate solution the drop approaches the temperature of the solution so rapidly that the effects of several degrees of initial difference are nearly eliminated. Thus when plasma at 16° was dropped from a syringe (small drop) into copper sulfate solution at 26°, the error in estimating the gravity of the plasma was

only 0.0004, or one-eighth of what it would have been if the drop had not moved its temperature towards that of the solution. When 5 ml. of blood at 37° were drawn from a vein into a 10 ml. syringe at 26°, and at once tested with copper sulfate solutions at 26°, the gravity of the blood was never found to be more than 0.0002 lighter than when the test was made after the blood had cooled to 26°.

Blood drawn from a vein into a syringe can be delivered at once from the needle of the syringe into standard solutions at 20°, or above, without error from temperature ordinarily exceeding 0.0003 in the gravity measurement. However, blood or plasma drawn into a medicine dropper and delivered into the copper sulfate solutions should be within 5° of the temperature of the solutions. Blood from a refrigerator should be warmed to room temperature before the gravity is measured.

If cold copper sulfate standard solutions are brought into a warmer room, let the solutions come to within 1° or 2° of room temperature and then shake vigorously for a few seconds before using them. This precaution is necessary for three reasons: (1) The difference in temperature between the solutions and the blood or plasma may exceed the permissible 5°. (2) If the solutions are used while they are warming, they may develop convection currents that interfere with the determinations. (3) The solutions when cold dissolve an amount of air that makes them supersaturated when they are warmed. The warming can be accelerated by immersing the standards in water a few degrees above room temperature. The solutions are shaken after warming to discharge the excess of dissolved air. Otherwise air bubbles might form as the solutions stand, and attach themselves to drops of blood or plasma dropped into the solutions.

As precautions against convection currents, do not leave the standard solutions near a stove, on a window-sill, etc. If a container is held in the hand during a determination, hold it only by the top in order to avoid warming the solution with the hand.

4. Renewal of Standard Solutions—A 100 ml. standard solution should be replaced by a fresh solution after receiving 50 drops of blood or plasma, if the error in measurement of blood or plasma gravity due to the standard is to be kept within ± 0.0001 gravity unit. Tests have shown that addition of 50 small drops, about 1.5 ml., of blood or plasma to 100 ml. of standard CuSO₄ solution, causes the gravity of the copper sulfate standard solution to decrease by about 0.0002 unit. The standard solutions prepared according to Table II are made 0.0001 gravity unit too high to give a correct average plasma or blood gravity when used fresh. After receiving 50 drops, the standards become 0.0001 gravity unit too low. The resultant error in blood or plasma gravity is in the direction opposite to the deviation of the standard, being -0.0001 with a fresh standard and ± 0.0001 with a standard that has received 50 drops. The ± 0.0001 error in

gravity corresponds to ± 0.04 gm. of protein per 100 ml. of plasma or ± 0.06 gm. of hemoglobin per 100 ml. of blood, which is ordinarily negligible.

It is desirable to paste on each standard tube or bottle a piece of cross-section paper containing a number of squares equal to the number of drops of blood or plasma that the solution can receive. Mark off with a pencil one square for each drop added, and replace the solution when all the squares have been marked off.

Rough controls can be made by adding 1.5 ml. of plasma to a 100 ml. standard of 1.027 gravity and 1.5 ml. of blood to a 100 ml. standard of 1.058 gravity. The amount of copper proteinate precipitate that settles to the bottom of the controls indicates the maximum permissible.

If a maximal error corresponding to +0.12 gm. of plasma protein, or +0.18 gm. of hemoglobin, per 100 ml., from overuse of the standard solutions is permissible, the number of drops of blood or plasma permissible is raised to 100 per 100 ml. of standard solution; the effect is to decrease the gravity of the standard by about 0.0004 unit before it is discarded, with a resultant error of +0.0003 in the last blood or plasma gravity measurements made.

If, for any reason, it is impossible to renew a standard solution after it has been used often enough to decrease its gravity by 0.0002 or 0.0004, the standard may be brought back to approximately its original gravity by adding 0.2 or 0.4 ml., respectively, of stock CuSO₄ solution per 100 ml. of standard. Or, if stock solution is not available, the standard may be relabeled; e.g., a 1.0270 standard is relabeled 1.0268 or 1.0266. The authors do not recommend either subterfuge, but they may be resorted to in an emergency.

Although a standard of 100 ml. volume should be replaced after receiving 50 or 100 drops of blood or plasma, only a few standards of a set need be replaced after 50 or 100 blood analyses, because only those within the normal range are used in most analyses. Standards outside the normal range, 1.025 to 1.029 for plasma and 1.052 to 1.064 for whole blood, will ordinarily require infrequent replacement.

- 5. Avoidance of Evaporation of Standard Solutions—Unless the standard solutions are tightly closed when not in use, evaporation may occur to such an extent that the blood and plasma gravity results obtained are erroneously low. The bottles or tubes of the standard solutions are opened only for the length of time required for determination and then are closed tightly. For each determination, only those standards are opened that are likely to be used.
- 6. Correction for Added Anticoagulants—No corrections are needed if heparin, 0.1 or 0.2 mg. per ml. of blood, is used.

For each mg. of the ammonium-potassium oxalate mixture added per ml.

of blood, subtract 0.0004 from the observed G_B and the observed G_P (see Table VIII). If a test-tube prepared with 5 mg. of ammonium-potassium oxalate receives the expected 5 ml. of blood, the correction to G_P and G_B is therefore 0.0004. Neglect of the 0.0004 correction would lead to an overestimation of the plasma proteins by 0.15 gm. per 100 ml. of plasma, and of hemoglobin by 0.1 gm. per 100 ml. of blood, errors which may for some purposes be neglected.

- If, however, the volume of blood placed in the oxalated test-tube prepared to receive 5 ml. is less than 5 ml., the oxalate concentrations will be greater, and the corrections to G_P and G_B will be as follows: for 4 ml. of added blood, -0.0005; for 3 ml., -0.0007; for 2 ml., -0.0010; for 1 ml., -0.0020.
- 7. Standard Solutions Carried to High Altitudes—Dr. R. A. Johnson (personal communication) reports that when the standard copper sulfate solutions are carried from a low to a high altitude they must be shaken before being used, to discharge the dissolved air with which they are supersaturated. Otherwise air bubbles may form, as when cold solutions are warmed, and interfere with the gravity measurements.
- 8. Material to Which Copper Sulfate Method Is Inapplicable—As mentioned before, heparinized blood, or its plasma, after standing over 18 hours in the ice box may become so altered in surface properties that drops will not break through the surface film of the copper sulfate solution.

Quigley and Muraschi (12) found that two sera which had been preserved for 5 years failed to form copper proteinate sacs when dropped into copper sulfate solutions. They attributed this behavior to the formation of amino acids in the sera, which contained 46 and 105 mg. of α -amino nitrogen, respectively, per 100 ml. by the ninhydrin-CO₂ method (13), about 10 and 25 times the normal amount. Addition of similar amounts of amino acids to normal serum had a similar effect.

The method is not applicable to solutions containing less than about 2 gm. of protein per 100 ml., hence cannot be used for transudates and exudates.

9. Material Other Than Blood and Plasma to Which Copper Sulfate Method Is Applicable—Presumably the procedure can be used with any aqueous solution containing enough protein to form an adequate copper proteinate sac. Dr. Eugene Opie (personal communication) has employed it with pieces of tissue to estimate changes in water content.

EXPERIMENTAL

Relation of Specific Gravity to Concentration in Copper Sulfate Solutions

Solutions were prepared by weight. Into 50 ml. flasks were weighed varying amounts of CuSO₄·5H₂O, the composition of which had been

checked by drying 3 gm. samples at 300° to constant weight. 40 ml. of water were then pipetted into each flask, and the flasks were weighed again. The flasks were stoppered and rotated by hand till the copper sulfate was dissolved.

For the density determinations a pycnometer of 12.8 ml. capacity of the Ostwald-Sprengel type, of Pyrex glass, was used. Its form was that shown in Fig. 4 with the following changes: The horizontal arms were each 40 mm. long; no cock was attached to A; an etched ring encircled the arm B at about 25 mm. from its end. The pycnometer filled with water or solution was placed in a stirred water bath at 25° ± 0.05° until constancy of the location of the meniscus in the marked arm showed that constant temperature had been reached, which required about 5 minutes. The meniscus was then brought to the mark by touching the outlet of the unmarked arm with a pointed strip of blotting paper. The meniscus was observed with a lens, and if it was not exactly on the mark, a correction of 0.7 c.mm. per mm. of displacement was made. The pycnometer was then wiped dry and weighed. During weighing the pycnometer was suspended from the balance hook at such an angle that the meniscus retreated about 15 mm. from the end of capillary A; with this precaution no loss of weight could be detected during 30 minutes in the balance case. To prevent error from static charge on the wiped pycnometer (which on a dry day could be several mg.) two copper plates painted with radium bromide to ionize the air were placed in the balance case. With these, static error disappeared within about 1 minute. Before weighing a series of copper sulfate solutions, the empty pycnometer was immersed in the bath, wiped, and weighed in the same manner. Before and after a series of weighings of copper sulfate solutions, water at 25° was weighed in the pycnometer. Weights checked by the Bureau of Standards were employed. Duplicate weighings usually agreed within 0.2 mg. Correction for the buoyant effect of air was made by adding, to the uncorrected weight of the fluid in the pycnometer, 1.06 mg. per ml. of fluid. Variations in the correction due to atmospheric conditions were made negligible by the daily check with water.

The results were plotted on large scale coordinate paper with values of the ratio, $R = (D_{25}^{25} - 1)$: C, as ordinates and D_{25}^{25} as abscissae, C being the gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per 100 gm. of solution. The small variation of R with the density made graphic estimation of R for a given density possible to 1 part per 5000, whence C could be calculated as $R/(D_{25}^{25} - 1)$. The results are given in Table III, which served as the key table for computation of Table II.

The values of D_4^{25} of Table III were compared with values interpolated from the data in the International Critical Tables (14), and showed agree-

ment within 1 part in 500 in the concentrations of copper sulfate of the indicated densities.

To check the accuracy of the procedure previously described for preparing standard solutions by dilution of the stock solution, volumes of the latter from 10 to 80 ml. were measured into 100 ml. flasks from pipettes calibrated to 1 per 1000 accuracy, and water was added to the 100 ml. mark in the manner described for preparation of standard solutions. The densities were measured by pycnometer, as described above, and interpolated on the curve obtained from solutions prepared by weight. The interpolation indicated agreement, with solutions prepared by weight,

Table III

Relation of Concentration to Density in Copper Sulfate Solutions at 25°

Densi	Density of standard solutions					
D 25 216		D.**	CuSO ₄ -5H ₂ O per 100 gm.	CuSO ₄ per 100 ml. of solution	Stock solution per 100 ml. standard solution	
Uncorrected for buoyant effect of air	Corrected for effect of air	= b 0.99707	solution	$= c \times d$	0 1590	
(a)	(b)	(c)	(d)	(e)	ഗ	
			gm.	gm.	ml.	
1.010	1.00999	1.00703	1.520	1.531	9.63	
20	1.01998	1699	3.031	3.082	19.38	
30	2997	2699	4.523	4.646	29.22	
40	3996	3695	6.001	6.222	39.13	
50	4995	4691	7.465	7.814	49.13	
60	5994	5683	8.910	9.415	59.21	
70	6993	6679	10.53	11.02	69.31	
80	7992	7675	11.74	12.64	79.50	
1.100	1.09990	1.09667	14.50	15.90	100.0	

^{*} The stock solution at 25° contains 0.1590 gm. of CuSO₄. 5H₂O per ml.

within the limits of error of the measurement; *i.e.*, the technique described for preparing the standard solutions does not involve measurable volume error from incomplete mixing during addition of the water.

Temperature Coefficients of Expansion of Plasma and Blood, and of Copper Sulfate Solutions of Similar Specific Gravities

That no temperature corrections are required in determinations of blood and plasma gravities by the copper sulfate method is due to the fact that blood and plasma have temperature coefficients nearly identical with those of copper sulfate solutions of similar gravities.

The coefficients of expansion of plasma, whole blood, and copper sulfate solutions were determined in a dilatometer of about 18 ml. capacity and of

the form shown in Fig. 4. The left arm is 4 cm. long and has an etched mark at A. The right arm, B, is 40 cm. long and has a scale indicating volumes of fluid held between points on the scale and mark A. Both arms are at the same level to equalize hydrostatic pressure and are joined by a solid glass bridge to give strength and rigidity. The scale on B is of sufficient length to read volume changes over the temperature range $10-40^{\circ}$. The scale on B was calibrated with mercury.

The apparatus was filled by dipping the end of A into the liquid (which was at room temperature, $22-26^{\circ}$) and applying suction to the end of B till the fluid meniscus lay approximately in the middle of the scale. A was

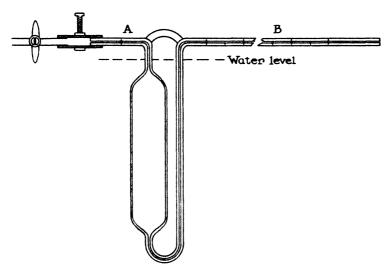


Fig. 4. Dilatometer for measurement of coefficient of expansion of fluids

then wiped dry and a short length of rubber tubing bearing a glass stop-cock and screw clamp, as shown in Fig. 4, was slipped over the end. When the cock was closed, the meniscus in A was adjusted to the etched mark by turning the screw clamp. The bulb of the dilatometer was then immersed in the center of a water bath of about 9 liters capacity to the depth shown by the dotted line, and supported so that the two arms were horizontal. A standard thermometer was placed alongside the dilatometer, with the center of the thermometer bulb at the same level as the center of the dilatometer bulb.

To determine the coefficients of expansion over the range 40-25°, the bath was warmed to 40° and maintained at that temperature for about an hour, till the fluid in the pycnometer had come to thermal equilibrium.

The heat was turned off and after about 15 minutes the meniscus in the arm A was adjusted to the mark, the volume was read off the scale on arm B, and the temperature was read on the thermometer. Temperature and volume were then read together every 20 to 30 minutes, the meniscus in A being adjusted to the etched mark when necessary, immediately before each reading; readings were continued till the temperature had fallen to 25° . For the range $10-25^{\circ}$, the bath containing the pycnometer was cooled by the addition of ice to 8° or 9° , maintained there for about an hour, and then allowed to warm up to room temperature. Readings of volume and temperature were started when the temperature had risen to 10° . About 6 hours were taken for the temperature of the bath to fall from 40° to 25° ,

Table IV

Coefficients of Expansion of Water, Plasma, Blood, and of Copper Sulfate Solutions of Specific Gravities Approximating Specific Gravities of Plasma and Blood

	Mean coeffic	cient of expansion of 1 ml. c	over temperature int aused by temperature	erval; ml. incres rise of 1°	ase in volume	
Temperature interval	Water	Plasma of D ₂₅ 1.0264	Copper sulfate solution of D_{2b}^{2b} 1.0270	Blood of D ₁₆ 1.0569	Copper sulfate solution of D_{25}^{35} 1.0597	
•c		1				
10-15	0.00012	0.00017	0.00016	0.00021	0.00021	
15-20	18	23	21	25	25	
20-25	24	27	26	29	29	
25-30	28	31	30	32	32	
30-35	33	35	34	36	36	
35–4 0	37	39	38	40	40	

and about the same interval to rise from 10° to 25°. Variations in room temperature were insufficient to cause, in the relatively small volume of liquid in the capillary arms, volume changes that were significant in relation to the changes of the total fluid in the dilatometer.

From the fact, shown in Table IV, that the technique gave, within the limits of its precision, temperature coefficients for water which agreed with those in the International Critical Tables, it appears that errors, from lag in temperature change of the dilatometer behind the change in the bath, and from other sources, were negligible in the fifth decimal place.

Non-Effect of Temperature on Results of Plasma and Blood Specific Gravity Determinations by Copper Sulfate Method

Specific gravity determinations were performed by the method described on a sample of blood and one of plasma at room temperature, and were then duplicated in rooms at 4° and at 38°, the samples and standard copper sulfate solutions being left in each room long enough to come to within 1° of its temperature. In order to check the readings to the fourth decimal place, after each gravity had been estimated to the fourth place by the usual interpolation method, with use of copper sulfate standards graded at intervals of 0.001, a mixture of the nearest two standards was made, such that the mixture had exactly the estimated blood or plasma gravity, and the blood or plasma was checked with this standard. Results are given in Table V.

Table V

Non-Effect of Temperature on Results of Plasma and Blood Gravities Estimated by

Copper Sulfate Method

perature	Gravities found by copper sulfate method			
Riature	Blood	Plasma		
°C	D ₂₅	D 25		
4	1.0585	1.0255		
23	1.0585	1.0252		
35	1.0588	1.0252		

Comparison of Blood and Plasma Gravities Measured by Pycnometer with Gravities Measured by Copper Sulfate and Gradient Tube Methods

The pycnometer measurements were made as described above for the copper sulfate solutions of Table III, except that the measurements were carried out in a room at approximately 25°, and a water bath was not used. All fillings and weighings were carried out in duplicate or triplicate. The temperature of the balance case was maintained usually at $25^{\circ} \pm 1^{\circ}$. If the temperature of the test solution was not identical with that of the water, the observed weight of the water was corrected for the temperature difference, which was always slight. The specific gravity was calculated directly from the weights as $D_{25}^{25} = \text{(weight of test solution) /(weight of water),}$ both weights being the apparent weights, uncorrected for the buoyant effect of air.

Duplicate weighings routinely agreed to ± 0.1 mg. Measurements of specific gravity routinely agreed within ± 0.00002 unit.

For the gradient tube method two 500 ml. cylinders were prepared as described by Lowry and Hunter (8), one for the range of plasma and one for whole blood. The only difference introduced from the technique of Lowry was that the standard copper sulfate solutions of D_{25}^{25} accurate to

 ± 0.0001 (Table III) were used to standardize the gradient tubes, instead of the potassium sulfate solutions used by Lowry. In order to assure maximal accuracy, when the gravity of a blood or plasma had been measured, the accuracy of the standardization curve of the gradient tube at that point was rechecked by 2 drops of copper sulfate solution within 0.001 of the determined gravity. Readings on the gradient tube were reproducible to ± 0.0001 , and deviations from true gravities exceeding this limit could be attributed to factors, such as absorption of bromobenzene by the drops of blood or plasma, other than the precision of the readings.

The copper sulfate method was carried out as described in the preceding pages, except that the standard solutions were graded at intervals of 0.0005, instead of the 0.0010 intervals used in routine analyses. The standards were prepared from Table III, not from Table II. The stock solution was prepared by weight from analyzed CuSO₄.5H₂O; both it and some of the standards were repeatedly checked by pycnometer and the D_{25}^{25} values were found exact within ± 0.0001 . Duplicate gravity estimations of the plasma and blood drops could be checked within 0.0001, and deviations from true gravities exceeding this limit could be attributed to factors other than the precision of the readings.

The results obtained with twenty-two samples of plasma are given in Table VI, and results with whole blood from twenty of the same subjects in Table VII. It is evident from comparison with the pycnometer values that readings by both the copper sulfate method and the gradient tube are slightly but measurably affected by sources of error other than the precision of the readings. In the copper sulfate readings, drops of plasma were balanced in solutions that averaged 0.0007 unit lighter than the plasma. When correction was made for this mean difference, the standard deviation of the corrected copper sulfate readings from the pycnometer values was ± 0.0003 . The gradient tube readings, on the other hand, indicated gravities averaging 0.0002 heavier than the pycnometer values; when correction was made for this mean difference, the standard deviation of the corrected gradient tube readings from the pycnometer values was ±0.0003, the same as for the corrected copper sulfate readings. The standard deviation of ± 0.0003 corresponds to ± 0.1 gm. of protein per 100 ml. of plasma.

Lowry and Hunter (8), working with serum (ten samples) and Hoch and Marrack (15) with plasma found that serum and plasma drops balanced in copper sulfate solutions that averaged 0.0010 lower than the plasma or serum gravity measured by pycnometer. Lowry and Hunter (8) found that their gradient tube method indicated serum gravities 0.0005 higher than by pycnometer. The deviations from pycnometer serum values indicated by our results are, for both the CuSO₄ and the gradient method, in

TABLE VI

Comparison of Plasma Gravities (Dm) Measured by Pycnometer, Gradient Tube, and

Copper Sulfate Methods

the same directions as Lowry and Hunter's but not quite so great. Hoch and Marrack suggest that enclosure of a film of air about the plasma drop

^{*} Measured as described by Lowry and Hunter (8) except that the calibration curve of the gradient column was made with drops of copper sulfate solution of known D_n^* instead of with drops of standard K_2SO_4 solution.

[†] Samples 1, 2, 4, 8, and 22 were from patients. The other seventeen plasmas were normal.

may cause it to appear lighter than the copper sulfate solution, and state that when they delivered the plasma under the solution, instead of letting the drop fall through the air, the difference disappeared.

Table VII

Comparison of Specific Gravities (D²⁵) of Whole Blood Measured by Pycnometer,
Gradient Tube, and Copper Sulfate Methods

		Values	of D_{25}^{25} m	easured	by three metho	ds		
		Copper sul	fate me	hod	Gradient tube			
Sample No.	Pycnometer	D25 of solution balancing blood	Deviation, $d = b - a$ (d)		Uncorrected*	Corrected by subtract- ing 0.0003	Deviation, $d = f - a$	
	(a)	(b)			(e)	(V)		(d)
2†	1.0464	1.0471	+0.	0007	1.0475	1.0472	+0.	0008
3	554	554		0	556	553	_	1
4†	493	490		3	497	494	+	1
5	566	564	_	2	566	563	_	3
6	534	536	+	2	538	535	+	1
7	587	580	_	7	582	579		4
8†	512	516	1 +	2	518	515	+	3
9	572	571	1 -	1	575	572		0
10	563	558	i –	5	563	560	_	3
11	581	584	+	3	583	580		1
12	605	607	+	2	608	605		0
13	605	600		5	606	603		2
14	599	599	1	0	601	598		1
15	589	592	+	3	592	589		0
16	5 99	605	+	6	609	606	+	7
17	537	540	+	3	539	536		1
18	537	541	+	4	543	540	+	3
19	57 9	585	+	6	581	578		1
20	551	550	_	1	552	549		2
21	563	558	_	5	563	560		3
lean deviation		+0.00004	0.0	0000	+0.00028	0.0000	0.	0000
and deviation $\sqrt{d^2/(N-d^2)}$	iation,		±0.0	00038			±0.	0003

[†] See the foot-notes of Table VI. The numbers in the left-hand column indicate bloods from which plasmas with the same numbers in Table VI were obtained.

Drops of whole blood (Table VII) balanced in copper sulfate solutions of the same average gravity as the blood. The consistent difference from the gravity of the balancing copper sulfate solutions, noted in plasma, is not apparent in whole blood.

Effects of Oxalates Added to Whole Blood on Specific Gravity of Blood and of Plasma Separated from Blood

Ammonium oxalate, potassium oxalate, and Heller and Paul's mixture (3 parts by weight of ammonium oxalate to 2 parts of potassium oxalate) were added to blood in amounts varying from 1 to 10 mg. per ml. The specific gravities of the bloods, and also of the plasma separated by centrifugation, were then measured by the copper sulfate method. The effects of the oxalates on the gravities were approximately linear functions of the

TABLE VIII

Changes in Gravity of Whole Blood and Plasma Caused by Addition of Ammonium
and Potassium Ozalates

	Extent of ch	xtent of change per mg. oxalate added to 1 ml. blood				
Change measured	Ammonium oxalate	Potassium oxalate	Oxalate mixture (3 gm. ammonium oxalate + 2 gm. potassium oxalate)			
Plasma gravity		+0.0002 +0.0006	+0.0003 +0.0004			
Plasma protein calculated from G_P^* Gm. per 100 ml. plasma	1	+0.07 +1.0	+0.11			
Hemoglobin calculated from G_P and G_B † Gm. per 100 ml. blood		+0.24 +1.5	+0.10 +0.6			

^{*} G_P = plasma gravity. For calculation of protein concentration from gravity, see the accompanying paper (9).

amounts added. The results, in terms of the effect per mg. of oxalate added per ml. of whole blood, are given in Table VIII.

The differences, shown in Table VIII, between ammonium and potassium oxalates with regard to their relative effects on plasma and whole blood gravities, respectively, are presumably attributable to differences in NH₄⁺ and K⁺ with regard to penetration into the cells. The ammonium-potassium oxalate mixture of Heller and Paul is chosen for routine use because its effects are about equal on plasma and whole blood gravities and on the plasma protein and hemoglobin values calculated therefrom.

 $[\]dagger G_B$ = whole blood gravity. For calculation of hemoglobin from G_B and G_P , see the accompanying paper (10).

SUMMARY

A simple technique is described for measuring the specific gravity of plasma, serum, or whole blood. The method is based on the facts that a drop of plasma or blood falling into a solution of copper sulfate of similar gravity is instantly encased in a sac of copper proteinate, and that the drop remains discrete and without change in gravity for about 10 seconds. During this interval the rise or fall of the drop indicates the relation of its gravity to that of the copper sulfate solution.

3 or 4 drops of blood or plasma and about a minute of time suffice for a gravity measurement. No temperature controls or corrections are needed, because the temperature coefficients of blood and plasma are so near those of the corresponding copper sulfate solutions that temperature changes between 10° and 40° do not significantly affect the gravity relations.

Drops of whole blood were found to balance in copper sulfate solutions of the same gravity. The standard deviation of blood gravity determined by the copper sulfate method was ± 0.0004 gravity unit from the blood gravity measured by pyenometer.

Drops of human plasma were found to balance in copper sulfate solutions averaging 0.0007 gravity unit lighter than the plasma. The standard copper sulfate solutions used for plasma are prepared to correct for this difference. The standard deviation of plasma gravity determined by the copper sulfate method was ± 0.0003 gravity unit from the plasma gravity measured by pycnometer.

BIBLIOGRAPHY

- Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., (a) Bur. Med. News Letter U. S. Navy, 1, No. 9, 1 (1943); (b) Reprinted with revision by the Josiah Macy, Jr., Foundation for the Armed Forces of the Allied Nations (1945).
- Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., Bull. U. S. Army Med. Dept., 71, 66 (1943).
- 3. Roy, C. S., J. Physiol., 5, p. ix (1884).
- 4. Rogers, L., Ann. Trop. Med. and Parasitol., 10, 129 (1906-07).
- 5. Barbour, H. G., and Hamilton, W. F., J. Biol. Chem., 69, 625 (1926).
- 6. Jacobsen, C. F., and Linderstrøm-Lang, K., Acta physiol. Scand., 1, 149 (1940).
- 7. Lowry, O. H., and Hastings, A. B., J. Biol. Chem., 143, 257 (1942).
- 8. Lowry, O. H., and Hunter, T. H., J. Biol. Chem., 159, 465 (1945).
- Van Slyke, D. D., Hiller, A., Phillips, R. A., Hamilton, P. B., Dole, V. P., Archibald, R. M., and Eder, H. A., J. Biol. Chem., 183, 331 (1950).
- Van Slyke, D. D., Phillips, R. A., Dole, V. P., Hamilton, P. B., Archibald, R. M., and Plazin, J., J. Biol. Chem., 183, 349 (1950).
- 11. Heller, V. G., and Paul, H., J. Lab. and Clin. Med., 19, 777 (1934).
- 12. Quigley, J. J., and Muraschi, T. F., J. Biol. Chem., 158, 463 (1945).
- 13. Hamilton, P. B., and Van Slyke, D. D., J. Biol. Chem., 150, 231 (1943).
- 14. International critical tables of numerical data, physics, chemistry and technology, New York, 3, 67 (1928).
- 15. Hoch, H., and Marrack, J., Brit. Med. J., 2, 153 (1945).

THE ESTIMATION OF PLASMA PROTEIN CONCENTRATION FROM PLASMA SPECIFIC GRAVITY

By DONALD D. VAN SLYKE,* ALMA HILLER,† ROBERT A. PHILLIPS,‡ PAUL B. HAMILTON,§ VINCENT P. DOLE, REGINALD M. ARCHIBALD, AND HOWARD A. EDER

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, July 18, 1949)

In 1930 Moore and Van Slyke (1) reviewed the various physical measurements, refractometric and others, that might serve as measures of the protein concentration of plasma, and concluded that specific gravity was the most reliable if application was required to pathological as well as normal plasmas. For plasma of nephritic patients they found that the relation between protein concentration and specific gravity, D_{20}^{20} , was expressed by the linear equation, $P = 343 \ (G - 1.0070)$, P representing protein concentration in gm. per 100 ml. and G the specific gravity, D_{20}^{20} . The constants 343 and 1.0070 were based on gravity measurements by pycnometer and on proteins calculated from Kjeldahl nitrogen as $6.25 \times N$.

A number of authors have since published modifications of the equation P = a(G - b), with differing constants based on gravities measured by several procedures, and on micro-Kjeldahl analyses also by various procedures. The procedures and the constants arrived at for the equation are indicated in Table I. The gravity method indicated as $CuSO_4$ is the procedure described in the preceding paper (2), but without the correction of 0.0007 for the difference between D_{25}^{25} of copper sulfate standards and the plasma drops that balance in them. The gradient column method is that of Linderstrøm-Lang in the modification described by Lowry and Hunter (3). The Barbour method is the "falling drop" method described by Barbour and Hamilton (4). In order to compare calculations by the different equations the protein concentration calculated by each equation for plasma which has a gravity of 1.0270 determined by pycnometer is indicated in the column "P calculated."

From a recently published experimental review of Kjeldahl procedures (13) it appears probable that the differences in the relation between specific gravity and Kjeldahl-determined protein nitrogen found by different investigators (last column of figures, Table I) may in part be due to the fact

^{*} Present address, Brookhaven National Laboratory, Upton, New York.

[†] Present address, Presbyterian Hospital, Chicago 12, Illinois.

[‡] Present address, United States Naval Medical Research Unit No. 3, care of the American Embassy, Cairo, Egypt.

[§] Present address, Alfred I. du Pont Institute, Wilmington, Delaware.

Table I

Variations of Equation P = a(G - b) Formulated by Different Investigators

Authors and plasma	Year	Specific gravity		Jalues a and b used	Value of lated for of G = by pycn	plasma 1.0270	Method used to de- termine proteins
401018		incende used	a	ь	G by method used	P calcu- lated	termine proteins
					D_t^t	gm. per 100 ml.	
Moore and Van Slyke (1)	1930						Micro-Kjeldahl, digested with
9 normal men Nephritic patients		Pycnometer			1.0270 1.0270	7.16 6.86	H ₃ PO ₄ , H ₂ SO ₄ , K ₂ S ₂ O ₈ until
Weech, Reeves, and Goettsch (5); dogs, mostly hy- poproteinemic	1 1	"	340	1.0069	1.0270	6.83	Micro-Kjeldahl, no details
	1943	CuSO ₄	325	1.0064	1.0263*	6.47	
Hoch and Marrack (7, 8); human, normal	1945	Pycnometer Gradient col- umn	1		1.0270 1.0271†	7.30 7.25	Micro-Kjeldahl, digested 1-3 hrs. with Cu
		CuSO ₄	364	1.0060	1.0260†	7.30	and Se cata- lysts
Lowry and Hunter (3); human	1945	Gradient col-	348	1.0069	1.0275†	7.08	Micro-Kjeldahl, no details
Lloyd et al. (9); human, normal	1945	<i>u u</i>	301	1.0027	1.0270†	7.32	Gravimetric
Meyer et al. (10); dogs	1945	Barbour CuSO ₄	1		1.0263 1.0263*	6.41 6.63	Micro-Kjeldahl, Cu and Se cat- alysts; di- gested 10 min.
Dicker (11); rats	1948	"	364	1.0060	1.0263*	7.39	Micro-Kjeldahl, Cu and Se cat- alysts; di- gested 5 hrs.
Lloyd et al. (12); human with famine edema	1948	Gradient col- umn	354	1.0073	1.0270†	6.97	Gravimetric

^{*} The authors quoted, following the usage of a preliminary report by the present writers, took the D_i^t of plasma as equal to the D_i^t of the CuSO₄ solution in which the plasma drop balanced. As shown in the preceding paper (2), by this procedure plasma D_i^t is estimated on the average as 1.0263 when the pycnometer value is 1.0270. Meyer et al. (10) in their measurements found the same gravities by the Barbour falling drop method as by the preliminary copper sulfate procedure; hence the same correction of 0.0007 is applied to their gravity measurement by the Barbour method.

 $[\]dagger$ G values by methods other than pycnometer, as found for plasmas with pycnometer G of 1.0270, by the authors cited.

that most of the Kjeldahl digestion procedures used give low results when applied to plasma protein. Hiller $et\ al.$ (13) found that nitrogen values equal to those yielded by Dumas combustion were obtained only when mercury and potassium sulfate were used as catalysts, or when digestion with copper and selenium as catalysts was prolonged for several hours. It will be noted in Table I that, for plasma of $D_t^i=1.0270$, the protein concentrations estimated by Hoch and Marrack and by Dicker, who digested for 1 to 5 hours, and by Lloyd $et\ al.$ (9), who determined the proteins gravimetrically, are higher than the concentrations estimated by authors who used shorter or unstated Kjeldahl digestions.

The work presented in this paper was carried out to ascertain with as much accuracy as possible the relation of plasma specific gravity to human plasma protein concentration calculated from nitrogen analyses.

Methods

Blood for the analyses was drawn and treated with heparin as described in the preceding paper (2). For the data on normal subjects in Table III blood samples of about 20 ml. were drawn; 10 ml. of the plasma were used for determination of gravity by pycnometer as described in the preceding paper (2). Portions were then used for check determinations of the gravity by the copper sulfate method (2), and for macro-Kjeldahl nitrogen determinations. For the data on hospital patients reported in Table II, portions of 5 ml. or less of blood were drawn, and gravities were determined by the copper sulfate method (2). In the determinations by the copper sulfate method, the gravity of the plasma is taken as 0.0007 unit above the gravity of the copper sulfate solution in which the plasma drops balance. (This correction is automatically made when the standard copper sulfate solutions are prepared according to Table II of the preceding paper (2).)

The methods used for total nitrogen determination are indicated in the headings of Tables II and III. The method of Hiller et al. (13), with Hg and K₂SO₄ as catalysts in the digestion, was found (13) to give nitrogen values for proteins equal to those obtained by Dumas combustion. The analyses by the Campbell-Hanna (14) method, with Cu and Se as catalysts, were performed before the more accurate procedure of Hiller et al. had been worked out. The Campbell-Hanna method was modified by prolonging the digestion for 2 hours after the digest cleared. In a series of analyses by both methods, the Campbell-Hanna procedure was found to yield 98.4 per cent as much nitrogen as the procedure of Hiller et al. (13) with mercury catalyst; hence nitrogen values obtained by the Campbell-Hanna method were multiplied by the correction factor 1.016.

Protein nitrogen was calculated by subtracting non-protein nitrogen (trichloroacetic acid filtrate N determined by gasometric micro-Kjeldahl (15)) from the total plasma nitrogen. The weight of protein was calculated as $6.25 \times N$, the factor 6.25 being that obtained by Hiller *et al.* (13) in analyses of fat- and ash-free preparations of normal human plasma protein.

Table II

Plasmas from Hospital Patients

G values all determined by the copper sulfate method (2).

	Miscellaneous hospital patients	Nephritic patients
No. of plasmas*	50	41
Type of Kjeldahl analysis used to determine P_N	Macro, with Cu and Se catalysts, 2 hrs digestion (14)†	Micro, with Hg and K ₂ SO ₄ catalysts (13)
Range of P_N , gm . per 100 ml.	5.62-12.44	3.71-8.03
Mean P_N , gm . per 100 ml .	7.282	4.732
" G	1.02697	1.01996
" value of b in equation $P_N = 365 (G - b)$	1.00702	1.00700
	gm. per 100 ml	gm per 100 ml.
Comparison of P_G calculated as 365 ($G-1.0070$) with P_N calculated as $6.25 \times N$		
Mean algebraic difference, $P_G - P_N$	+0.014	± 0.000
Standard deviation of P_q from P_N	± 0.22	± 0.26
Maximal + " " " "	+0.58	+0.53
	-0.32	-0.52

^{*} Plasmas with non-protein nitrogen over 100 mg. per 100 ml. are excluded from this table, and reported in Table V. In some cases two or more plasmas were drawn from the same patient on different dates.

Symbols

The following symbols are used. P = gm. of protein per 100 ml. of plasma. G = specific gravity of plasma in terms of D_{25}^{25} , the density of water at the same temperature as that of the plasma being taken as unity $(0.997G = \text{the density } D_4^{25})$. When it is desirable to distinguish between proteins calculated from nitrogen determinations and proteins calculated from plasma specific gravities, P_N is used to indicate the former and P_G the latter. V_P indicates the "apparent specific volume" of the proteins in

[†] The nitrogen values obtained by the digestion with Campbell and Hanna's (14) mixture of H₂SO₄, H₂PO₄, Cu, and Se were multiplied by the factor 1.016, to correct for the fact that this digestion was found to give 1.6 per cent less ammonia than the digestion with Hg and K₂SO₄ (13).

TABLE III Plasmas of Normal Adults

Range of normal specific gravity and protein concentration. Constants of equation, P = a(G - b). Comparison of protein concentration, P_N , calculated from Kjeldahl nitrogen, with concentration, P_G , calculated from plasma gravity.

	Group I, 1	7 subjects	Group II, 20 subjects
Type of Kjeldahl analysis used to determine P_N .	Macro, with Hg and K ₂ SO ₄ catalysts, 2 hrs. digestion (13), samples of 2 ml. plasma		Macro, with Cu and Se (14), 2 hrs. digestion*
Range of normal P_N calculated as $6.25 \times \text{protein N}$			
Mean, gm. per 100 ml.	7.39		7.38
s.D. from mean, gm. per 100 ml.	±0.34		±0.28
Maximum observed, gm. per 100 ml.	8.03		8.17
Minimum " " 100 "	6.76		7.05
•	G by pyc- nometer	G by CuSO ₄ method	G by CuSO4 method
Range of normal gravity			
Mean G	1.02683	1.02681	1.02678
S.D. from mean	±0.0099	±0.0091	± 0.0086
Maximum observed G	1.0287	1.0283	1.0285
Minimum " "	1 0247	1.0249	1.0252
Mean value of a calculated as			
$a = \frac{P_N}{(\bar{G} - 1\bar{0}070)}$	372.7	373.0	373.1
	gm. per 100 ml	gm per 100 ml.	gm. per 100 ml.
('omparison of P_G calculated as 373 ($G-1$ 0070) with P_N calculated as 6 25 \times N			
Mean algebraic difference, $P_G - P_N$	+0.007	±0.000	-0.002
s.p. of P_a from P_N	± 0.093	± 0.106	± 0.136
Maximal + deviation of P_a from P_N	+0.14	+0.15	+0.17
	-0.15	-0.25	-0.31

^{*} See foot-note (†), Table II.

plasma, defined as the volume of solvent (water + non-protein solutes) that is displaced by 1 gm. of protein.

Estimation of Constants of Equation,
$$P = a(G - b)$$

The range of protein concentrations in normal plasmas is too narrow to indicate closely the best values for the constants. Thus a and b respec-

¹ For a discussion of "apparent partial specific volumes" of proteins in solution, see Kraemer (16).

tively may be taken for normal plasma as 360 and 1.0063, 370 and 1.0069, or 380 and 1.0074, without a statistically significant difference in the accuracy of the equation. Consequently we have used the wider protein concentration range of pathological plasmas to estimate the best line (Fig. 1) which is defined by Equation 1 (Table IV).

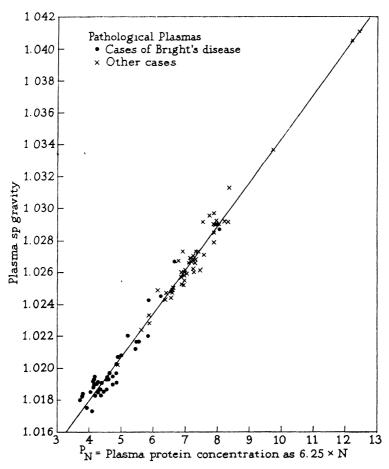


Fig. 1. Relation of specific gravity to nitrogen-calculated protein in pathological plasmas.

When this equation was used to calculate proteins from gravities for the normal plasmas of Table III, the resulting P_{σ} values averaged 2.2 per cent lower than the P_{N} values. The difference is indicated by the greater a value of Equation 2 (Tables III and IV), b being kept constant at 1.0070. Equation 3 (Table IV) is used for computing the nomograms for general

use in the following paper (17). Equation 3 gives on the average plasma protein concentrations 1 per cent lower than nitrogen-calculated values of the normal plasmas of Table III, and 1 per cent higher than the nitrogen-calculated values of the pathological plasmas of Table II.

Factors Determining Constant a in Equation P = a(G - b)—From the equation it is evident that 1/a represents the increase in gravity per increase of 1 gm. per 100 ml. in protein concentration. An a of 373 indicates that an increase of 1 gm. per 100 ml. in P causes an increase of 1/373 = 0.00268 in G, owing to replacement of solvent (water + non-protein solutes) of gravity b by protein of greater density.

The quantitative relation of a to the specific volume of the dissolved proteins may be calculated as follows: Increase of 1 gm. per 100 ml. in P displaces V_P ml. of solvent of density 0.997b. The weight of solvent displaced is $0.997bV_P$ (density \times volume). The resultant increment of weight per 100 ml. of plasma is $1 - 0.997bV_P$, and the increment in weight per

Table IV

Equations for Calculating Plasma Protein Concentration, P, from Specific Gravity, G

Equation No.	Type of plasma Formula to calculate protein, P, from gravity, G	
1	Pathological	P = 365(G - 1.0070)
2	Normal	"=373("-1.0070)
3	" and pathological	" = 369(" - 1.0070)

1 ml., or in D_4^{25} , is $(1 - 0.997bV_P)/100$. Since $D_4^{25} = 0.997D_{25}^{25} = 0.997G$, the increment in G caused by an increase of 1 gm. in P is $(1 - 0.997bV_P)/99.7$. This increment is 1/a. Hence

$$a = \frac{99.7}{1 - 0.997bV_P}$$

Rearranging to calculate V_P gives

$$V_P = \frac{a - 99.7}{0.997ab}$$

From the a and b values of Equation 2, for normal plasmas, one calculates $V_P = 0.730$. Hence an increase of 1 gm. per 100 ml. in P involves the displacement of 0.730 ml., or $0.730 \times 1.0070 \times 0.997 = 0.7329$ gm., of solvent, and an increase of 1 - 0.7329 = 0.2671 gm. in the weight of 100 ml. of plasma. There results an increase of 0.002671 in the density D_4^{25} , and an increase of 0.002671/0.997 = 0.002679 in D_{25}^{25} .

From observations of Adair and Adair (18), it appears that proteins, unlike most crystalloid solutes, may dissolve without measurable shrinkage in volume, the volume of the solution formed equaling the volume of the solvent plus the volume of the dry protein. In the case of edestin and hemoglobin the apparent specific volumes of both proteins were found to be 0.744 ± 0.002 in aqueous solution, and the specific volume (1/(density)) in the dry state was also found to be 0.744 ± 0.002 , from density values of 1.345 ± 0.003 . The apparent behavior of the proteins in dissolving without volume change is consistent with the linear form of the equation, P = a(G - b).

From the a of 365 of Equation 1, one calculates for the pathological plasmas of Table II a V_P of 0.724, smaller than the V_P of 0.730 calculated for normal plasma. Pedersen (19) found for human albumin a V_P of 0.736, for γ -globulin, 0.718. It consequently appears possible that changes in the ratio of γ -globulin to albumin could cause changes in V_P of the order required to cause the average difference of 2 per cent in P_N between the normal and pathological plasmas of the same gravity in our series.

For a protein, fetuin, from the plasma of a new-born calf, Pedersen (19) found a V_P of 0.70, suggesting that the presence of abnormal proteins, as well as changes in the distribution of normal ones, may influence V_P and hence the a value of the equation P = a(G - b).

Fig. 2 shows no correlation between the A:G ratio, determined by the Howe method, and the $P_{\sigma}-P_{N}$ difference. Present data do not suffice to show whether correlation would become evident if the γ -globulin fraction was determined separately, and if the influence of other factors, such as error in calculating protein as $6.25 \times N$ in pathological plasma, were eliminated.

Factors Determining Constant b in Equation P = a(G - b)—The value 1.0070 of the constant b approximates the gravity of a solution of the plasma crystalloids. This was demonstrated by an experiment in which normal plasma was filtered under pressure through cellophane. Filtrates, free of protein, showed by micro pycnometer D_{25}^{26} values approximating 1.0070 within the limit of error, ± 0.0003 , of the measurement.

Per unit concentration, the increment in gravity caused by the organic crystalloids is of the order of the increment caused by protein (0.0027), while the mineral constituents cause from 2 to 2.5 times as great increments. Per gm. per 100 ml., the increments caused by the chief plasma crystalloids are as follows: urea 0.0029, glucose 0.0039, NaCl 0.0071, NaHCO₃ 0.0062, KCl 0.0064, CaCl₂ 0.0087. The total increment in gravity due to the crystalloid solutes of normal plasma may be approximately estimated by assuming that of the total mineral salts, indicated by a total base of 154 m.eq. per liter, 105 m.eq. have the effect of NaCl and the remainder the

effect of NaHCO₃, and that the effects of the nitrogenous crystalloids approximate that calculated by assuming that all the non-protein nitrogen is urea. The respective gravity increments caused by the solutes thus calculated are NaCl 0.0036, other mineral salts 0.0025, glucose (100 mg. per 100 ml.) 0.0004, non-protein nitrogenous solutes (30 mg. of non-protein N per 100 ml.) 0.0002, total increment 0.0067.

The lipides, as non-protein constituents, could affect the value of b if they altered significantly the gravity of the non-protein phase of the plasma. From the fact that fats in general are lighter than water, one might expect their presence in plasma to lower the gravity of the non-protein phase, and

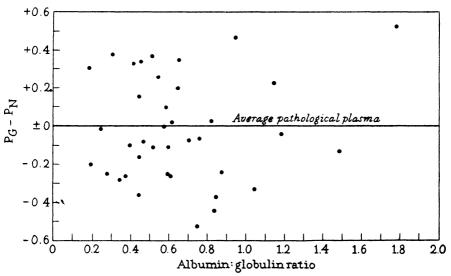


Fig. 2. Lack of correlation between the albumin-globulin ratio and the $P_G - P_N$ difference in nephrotic plasmas.

to cause markedly negative errors in gravity-calculated proteins when the plasma lipides are high. However, Popjak and McCarthy (20) found that the mean apparent partial specific volume of the lipides in plasmas examined by them was 0.996, indicating a value of 1.004 for the density, D_4^{25} , of the lipide phase; this corresponds to a D_{25}^{25} of 1.0070, identical with the b value, assumed in Table IV as the D_{25}^{25} of the non-protein phase of plasma. In part, the apparent high density of the lipide phase, compared with ordinary fat, is attributable to the fact that about 40 per cent of the mixed plasma lipides ordinarily consists of cholesterol, which has a density of 1.067. It is possible also that the loose combination of the lipides with proteins, which makes varying amounts of lipides invisible in plasma, may

decrease the apparent specific volume of the lipides. The plasma lipides, at least up to the maximal concentrations (about 3 gm. per 100 ml.) studied by Popjak and McCarthy (20), appear to have little effect on the relation of plasma gravity to protein concentration.

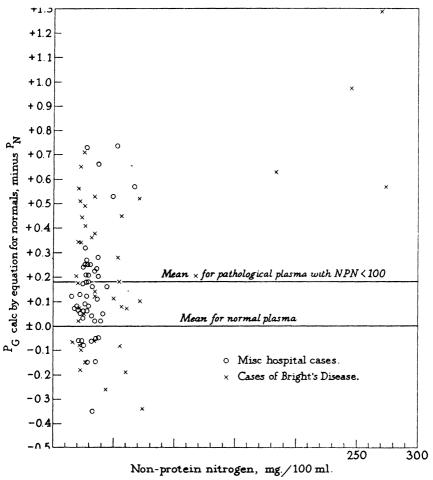


Fig. 3. Effect of high plasma non-protein nitrogen concentrations on the $P_G - P_N$ differences in pathological plasmas.

In Table II a large proportion of the subjects in the group with Bright's disease were lipemic, but this group, like the miscellaneous pathological group in the same table, showed P_o values that, if calculated by the equation for normal plasma, averaged 2 per cent *above* the nitrogen-calculated proteins (see Figs. 2 and 3).

In exceptional plasma, with lipemia so gross that a large layer of fat could be removed by centrifugation, an increase in plasma gravity as the result of centrifugation has been observed of an order to indicate that the material removed had a D_{25}^{25} less than 1.007; but even in such cases, some of which are included in Fig. 2, the effect of the lipides on P_a did not lower the latter by more than 0.2 gm. per liter.

Sources of Error in Calculation of Weights of Proteins As 6.25 × N in Pathological Plasma

Part of the deviation of P_g from P_N noted in Table II is probably due to error, not in P_0 , but in P_N calculated as $6.25 \times N$. The factor, 6.25, is based on an assumed constant protein nitrogen content of 16.0 per cent, found in analyses of fat- and ash-free total proteins of normal human plasma The different fractions of normal human plasma, analyzed by Dumas combustion by Brand, Kassell, and Saidel (21), had the following percentages of nitrogen: albumin 15.95, β-globulin 14.84, γ-globulin 16.03, fibringen 16.90. It is evident that either abnormalities in the proportions in which the different normal fractions are present in the total plasma protein mixture, or the presence of abnormal proteins, may significantly alter the percentage of nitrogen in the mixture, and the accuracy of the calculation of gm. of protein as 6.25 × gm. of N. Elimination of variation in protein nitrogen content as a source of error in chemically determined proteins could be accomplished by determining the proteins gravimetrically (22), as has been done by Lloyd et al. (12) in cases of famine edema, or by wet carbon combustion of the lipide-free proteins by the method of Hoagland and Fischer (23).

A relatively small plus error is introduced into P_N by the nitrogen content of the phosphatides. The aqueous protein precipitants ordinarily used, such as trichloroacetic and tungstic acid solutions, form protein precipitates which carry down with them all of the lipides of plasma. Hence in the usual procedures for determining protein nitrogen, including that of the present papers, the nitrogen of the phosphatides is included. The approximate effect may be estimated as follows: The phosphatides comprise about 25 per cent of the total lipides. The nitrogen content of the phosphatides is about 2 per cent; hence the nitrogen content of the total lipides is about 0.5 per cent. An increase in plasma lipides of 3 gm. per 100 ml. would increase the apparent P_N by about 0.1 gm. per 100 ml., and make the gravity-calculated proteins appear by comparison to be that much too low. The error in P_N caused by lipide nitrogen is negligible (about 0.02 gm. per 100 ml.) in normal plasma, but may become significant in gross lipemia. Such an effect could be a factor in some of the relatively few

cases in Figs. 2 and 3 in which P_G calculated by the equation for normal plasma falls below P_N .

Magnitude of Intrinsic Error in Calculation of Proteins from Gravity in Normal and Pathological Plasmas

By intrinsic error is meant error in excess of that due to error in measurement of the specific gravity; it is the error due to variation of a and b in the equation P = a(G - b) from their assumed values. To estimate the magnitude of the maximal intrinsic error from results in Tables II and III, we subtract from the observed maximal $P_a - P_N$ difference the sum of the estimated effects of maximal errors in the gravity and the Kjeldahl methods used, and obtain a result which is presumably larger than the true intrinsic error of P_a (because error in P_N due to variation from the factor 6.25 is ignored), but is the best estimate that can be made at present of the intrinsic error.

As maximal experimental errors in Tables II and III we will assume ± 0.0001 for gravities measured by pycnometer, ± 0.0006 for gravities measured by the copper sulfate method (2), and ± 0.5 per cent of the nitrogen determined by Kjeldahl analysis (13). The gravity errors of the two methods correspond to errors of ± 0.04 and ± 0.22 gm. respectively of protein per 100 ml. of plasma. The nitrogen error, for a plasma of mean normal protein content, corresponds to ± 0.04 gm. of protein per 100 ml. The combined maximal experimental errors of P_{σ} and P_{N} thus estimated are ± 0.08 gm. of protein per 100 ml. of plasma when gravities are measured by pycnometer and ± 0.26 gm. when gravities are measured by the copper sulfate method. Errors in P_{N} that may be due to deviations from the factor 6.25 are not included in the estimates.

Normal Plasma—In the three groups of observations in Table III, the maximal deviations of P_{σ} from P_{N} , calculated as twice the standard deviations, are ± 0.19 , ± 0.21 , and ± 0.27 gm. per 100 ml., compared with estimated maximal experimental errors of ± 0.08 , ± 0.26 , and ± 0.26 gm. per 100 ml., respectively. The observed standard deviation \times 2 exceeds the estimated experimental errors by 0.11 gm. per 100 ml. in the first group, falls short by 0.05 in the second, exceeds by 0.01 in the third.

It appears probable that the intrinsic error in the calculation of protein concentrations in normal human plasma from gravities by Equation 2 does not exceed 0.1 gm. per 100 ml. It also appears that deviations of the protein nitrogen content from 16.0 per cent in normal plasma are small; otherwise error in P_N from such variation would have caused the deviation of P_σ from P_N to exceed more definitely the deviation predictable from the errors in gravity measurement and Kjeldahl analysis.

Pathological Plasmas—In the pathological plasmas of Table II, the maxi-

mal deviations of P_{σ} from P_{N} , calculated as twice the standard deviations, are 0.44 and 0.52 gm. per 100 ml. in the two groups. These definitely exceed the 0.26 gm. difference estimated as the maximum attributable to errors in the Kjeldahl nitrogens and in the gravities determined by the copper sulfate method. The intrinsic error of gravity-estimated proteins in pathological plasmas of the types covered by the cases in Table II would accordingly be estimated at about 0.2 gm. per 100 ml., if the P_{N} values could be assumed to be without error from use of the factor 6.25. Since there is reason to assume that part of the deviation of P_{σ} from P_{N} in pathological plasmas may be due to error in the factor 6.25, it appears probable that the maximal intrinsic error of gravity-calculated proteins in pathological plasmas of the type in Table II (without non-protein N over 100 mg. or very high blood sugar) is something less than 0.2 gm. per 100 ml.

More extensive data, with all gravities precise to ± 0.0001 unit, and with chemical determinations of the proteins by a method not dependent on an assumed constant nitrogen content, would provide more satisfactory statistics for estimation of the intrinsic error of P_G . The above estimates, however, appear to provide a fair approximation of the accuracy with which proteins can be calculated in human plasma from gravities measured without significant error.

Probable Causes of Greater Deviations of Gravity-Calculated from Nitrogen-Calculated Proteins in Pathological Than in Normal Plasmas—In cases of gross glucemia, or such high blood non-protein nitrogen concentrations as are exemplified in Table V, abnormally high crystalloid concentrations can cause gravity-calculated proteins to be too high.

If cases with such gross crystalloid increases (Table II) are excluded, however, pathological plasmas show a variability in the relation of gravity-calculated to nitrogen-calculated proteins that appears to be due to variations in either the nitrogen contents or specific volumes of the proteins themselves. The fact that, for a given specific gravity, the average plasma protein content was 2 per cent less in the pathological plasmas of Table II than in the normal plasmas of Table I could be due either to lower nitrogen content (15.7 versus 16.0 per cent) or lower specific volume (0.724 versus 0.730) of the proteins of the pathological group.

Some of the results in the literature appear to indicate also that, in conditions of malnutrition, abnormalities in the plasma proteins similarly affect the $P_{\sigma} - P_{N}$ difference. Hoch and Marrack (8) observed that, in plasmas from a group of subjects suffering from malnutrition, the nitrogencalculated protein content averaged 0.54 gm. per 100 ml. lower than in normal plasmas of the same specific gravity, the abnormality being in the same direction observed in the pathological plasmas of Table II. Because

ase No.

1

2

3

4

1.0249

6.53

there is no reason to suspect the presence of such gross increases of crystal-loid solutes (e.g. 400 mg. of glucose or 500 mg. of urea per 100 ml. above normal) as would be necessary to cause the observed effect in Hoch and Marrack's subjects, it appears that the observed $P_{\sigma} - P_{\nu}$ difference may have been due to abnormality in the proteins. The abnormal relation between P_{ν} and P_{σ} in the malnourished group could be caused either by a decrease in specific volume of the proteins from the 0.730 of normal plasma proteins to 0.706 in the malnourished group, or by a decrease in nitrogen content from the 16 per cent in normal proteins to 14.8 per cent in the malnourished group, or by smaller shifts in both values.

Error of PG $P_G = 365 \times$ $P_N = 6.25 \times$ G by CuSO₄ method Difference, estimated by Non-protein assuming excess (G - 1.0070)protein N $P_G - P_N$ non-protein N* is urea (a) (b) (d) (e) (c) gm. per 100 ml. gm. per 100 ml. gm. per 100 ml. gm. per 100 ml gm. per 100 ml. 1.02807.67 6.63 +1.040.272+0.556.280.249+0.501.0242 5.58+0.706.79 1.02566.48 +0.310.275+0.55

+0.36

0.185

+0.35

TABLE V
Cases with Non-Protein Nitrogen Greater Than 100

6.17

A similar difference from normal subjects (9) in subjects with famine edema (12) was noted by Lloyd $et\ al$. (see Table I). Lloyd $et\ al$. compared the gravities of the plasmas of their famine group with protein concentrations determined, both gravimetrically, as described by Robinson and Hogden (22), and by Kjeldahl, and concluded that both factors, low protein specific volume and low protein nitrogen content, contributed to the positive value of the $P_{\sigma}-P_{N}$ difference. Gravimetrically determined protein concentrations averaged 0.35 gm. per 100 ml. lower in plasma of the same gravity in the famine-stricken than in the normal group (compare data of Lloyd $et\ al$. for 1945 and 1948, Table I); this difference, being independent of nitrogen values, may be attributable to smaller specific volume (greater density) of the proteins in the starved group. If this is the case, the equation P = a(G-b) would require, for starved subjects, an a value still smaller than that of Equation 1 (Table IV).

^{*} Excess non-protein nitrogen is taken as the amount above 0.030 gm. per 100 ml. The increment of G per gm. of urea per 100 ml. is taken as 0.0029 (see p. 338), and hence as 0.00621 per gm. of urea N per 100 ml. The effect of "excess non-protein N" on G is hence estimated as a G increment of 0.00621 \times "excess non-protein N," and the error in P_G caused by non-protein N is taken as $365(0.00621 \times$ "excess non-protein N") = $2.27 \times$ "excess non-protein N."

Apparent Partial Specific Volume of Proteins in Normal Human Plasma¹

The data of Table III permit calculation by Equation 5 of the partial specific volume of the proteins in normal plasma from the a and b values of the equation P = a(G - b). As pointed out, the a and b values may be varied considerably without a statistically significant change in the accuracy of the equation for calculating P. However, it appears that the correct value for a in normal plasma, with G measured by pycnometer, is within the range 373 \pm 15, with the corresponding b in the range 1.0070 \pm 0.0009. The corresponding V_P range calculated by Equation 5 from these a and b values is 0.730 ± 0.010 . This is not out of line with Pedersen's (19) values of 0.736 for human albumin and 0.718 for γ -globulin. which together constitute about 70 per cent of normal human plasma protein, or with Popjak and McCarthy's (20) value of 0.729 for rabbit proteins. In earlier (1928) work on the proteins of horse serum, Svedberg and Sjögren (24) found higher and nearly identical values, 0.748 for the albumin fraction and 0.745 for the total globulins, however, they determined their protein concentrations by weighing the heat-coagulated proteins, which may have contained some occluded fat.

Range of Normal Plasma Protein Concentration—The data of Table III indicate 7.4 ± 0.6 gm. per 100 ml. as the normal range. The data were obtained by macro-Kjeldahl analyses performed with precautions for accuracy (13), and presumably indicate the approximate range for plasma drawn from the blood of normal healthy young adults in the standing or sitting position. As a number of authors have shown, however (cf. Lange (25)), change from the upright to the horizontal position causes a decrease in plasma protein concentration, which is measurable in a few minutes and approaches a plateau in about an hour. It is due to dilution of the blood by interstitial fluid. Lange found the average protein concentrations in plasma of blood drawn from subjects lying down to be 0.55 gm. per 100 ml. lower than in plasma drawn while the subjects were standing. For subjects in bed, therefore, a range of about 6.8 ± 0.6 gm. per 100 ml. may be normal.

SUMMARY

From comparison of plasma specific gravities with protein concentrations calculated from accurate Kjeldahl analyses as N \times 6.25 the following equations have been derived: from thirty-seven normal plasmas, P=373~(G-1.0070); from 91 pathological plasmas without gross elevation of blood urea or sugar, P=365~(G-1.0070). P indicates the gm. of protein per 100 ml. of plasma, G the specific gravity of the plasma, the density of water at the same temperature being taken as unity.

As indicated by the difference between the constants, 373 and 365, the pathological plasmas had on the average 0.98 as much nitrogen-calculated protein per 100 ml. as normal plasmas of the same specific gravity. Data from the literature indicate that plasma of subjects suffering from malnutrition shows a similar difference from normal plasma. The difference could be due to either lower specific volume or lower nitrogen content of the proteins of the abnormal plasmas.

It is estimated that, with errors of gravity measurement eliminated, the error in calculating protein concentration from gravity is less than 0.1 gm. per 100 ml. in normal human plasma, and less than 0.2 gm. in pathological plasma without gross abnormalities of crystalloid concentration, such as sugar or urea concentrations above 200 mg. per 100 ml.

The range of nitrogen-calculated protein concentration observed in plasma of blood drawn from healthy men in the upright position was 6.8 to 8.0 gm. per 100 ml., average 7.39.

The apparent specific volume of the proteins in normal human plasma is estimated at 0.730 ± 0.010 .

BIBLIOGRAPHY

- 1. Moore, N. S., and Van Slyke, D. D., J. Clin. Invest., 8, 337 (1930).
- Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., J. Biol. Chem., 183, 305 (1950).
- 3. Lowry, O. H., and Hunter, T. H., J. Biol. Chem., 159, 465 (1945).
- 4. Barbour, H. G., and Hamilton, W. F., J. Biol. Chem., 69, 625 (1926).
- 5. Weech, A. A., Reeves, E. B., and Goettsch, E., J. Biol. Chem., 113, 167 (1936).
- Cole, W. H., Allison, J. B., and Boyden, A. A., Proc. Soc. Exp. Biol. and Med., 54, 215 (1943).
- 7. Hoch, H., and Marrack, J., Brit. Med. J., 2, 151 (1945).
- 8. Hoch, H., and Marrack, J., Brit. Med. J., 2, 876 (1945).
- Lloyd, B. B., Cheek, E. B., Sinclair, A. M., and Webster, G. R., Biochem. J., 39, p. xxv (1945).
- Meyer, F. L., Abbott, W. E., Allison, M., and McKay, C., Arch. Biochem., 12, 359 (1947).
- 11. Dicker, S. E., J. Physiol., 107, 11P (1948).
- Lloyd, B. B., Sinclair, H. M., and Tweedy, M. C. K., Biochem. J., 43, p. xvi (1948).
- 13. Hiller, A., Plazin, J., and Van Slyke, D. D., J. Biol. Chem., 176, 1401 (1948).
- 14. Campbell, W. R., and Hanna, M. I., J. Biol. Chem., 119, 1 (1937).
- 15. Van Slyke, D. D., J. Biol. Chem., 71, 235 (1926-27).
- Kraemer, E. O., in Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford, 59 (1940).
- Van Slyke, D. D., Phillips, R. A., Dole, V. P., Hamilton, P. B., Archibald, R. M., and Plazin, J. P., J. Biol. Chem., 183, 349 (1950).
- 18. Adair, G. S., and Adair, M. E., Proc. Roy. Soc. London, Series B, 120, 422 (1936).
- 19. Pedersen, K. O., Ultracentrifugal studies of serum and serum fractions, Upsala (1945).

VAN SLYKE, HILLER, PHILLIPS, HAMILTON, DOLE, ARCHIBALD, AND EDER

- 20. Popjak, G., and McCarthy, E. F., Biochem. J., 40, 789 (1946).
- 21. Brand, E., Kassell, B., and Saidel, L., J. Clin. Invest., 23, 437 (1944).
- 22. Robinson, H. W., and Hogden, C. G., J. Biol. Chem., 140, 853 (1941).
- Hoagland, C. L., and Fischer, D. J., Proc. Soc. Exp. Biol. and Med., 40, 581 (1939).
- 24. Svedberg, T., and Sjögren, B., J. Am. Chem. Soc., 50, 3318 (1928).
- 25. Lange, H., Acta med. Scand., suppl. 176 (1946).

CALCULATION OF HEMOGLOBIN FROM BLOOD SPECIFIC GRAVITIES

By DONALD D. VAN SLYKE,* ROBERT A. PHILLIPS,† VINCENT P. DOLE, PAUL B. HAMILTON,‡ REGINALD M. ARCHIBALD, AND JOHN PLAZIN*

(From the Hospital of The Rockefeller Institute for Medical Research, New York)
(Received for publication, July 18, 1949)

The specific gravity of the red blood cells (normally about 1.10) so greatly exceeds that of the plasma that the difference between the gravities of whole blood and plasma can serve as a measure of the cell content of the blood. By assuming a constant hemoglobin concentration in the cells, one can estimate also the blood hemoglobin concentration from the gravity values.

The calculation is based on two assumed constants; viz., the specific gravity of the red cells and the hemoglobin concentration of the red cells. Neither of these values is in fact constant. In some abnormal types of blood cells, hemoglobin concentration may fall 20 per cent below normal, with cell specific gravity showing a parallel decrease. However, changes in cell hemoglobin concentration and cell specific gravity parallel each other in such a way that their variations almost cancel each other in their effect on the relation of blood hemoglobin concentration to the concentration calculated from the gravities of whole blood and plasma. In consequence the error, even in grossly abnormal blood, is estimated to be only about 0.15 gm. of hemoglobin per 100 ml. of blood greater than the error attributable to the limit of accuracy of the gravity method used.

Ashworth and Tigertt (1) first formulated the equation for calculating blood hemoglobin concentration from the specific gravities of whole blood and plasma. The present paper reports tests of the accuracy of gravity-calculated hemoglobin concentrations by comparison with hemoglobin values determined by precise gasometric methods in normal and abnormal human blood. The gravity determinations have been made by the copper sulfate method (2). A nomogram (Fig. 1) is presented for rapid calculation of hemoglobin from the whole blood and plasma gravities.

Formulation of Equations for Hemoglobin Calculation

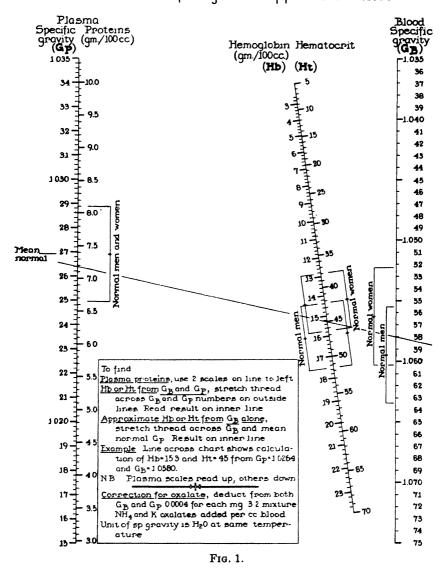
Let D_B , D_C , and D_G be the densities (D_4^t) of whole blood, plasma, and red cells, respectively. Let G_B , G_P , and G_C be the corresponding specific

^{*} Present address, Brookhaven National Laboratory, Upton, New York.

[†] Present address, United States Naval Medical Research Unit No. 3, care of the American Embassy, Cairo, Egypt.

[†] Present address, Alfred I. du Pont Institute, Wilmington, Delaware.

Line chart for calculating plasma proteins, hemoglobin and hematocrit from gravities of plasma and blood



gravities in terms of D_t^t . Let V_c be the volume of red cells in 1 volume of blood. Let Hb_s be the gm. of hemoglobin in 100 ml. of blood, and Hb_c the hemoglobin in 100 ml. of red cells. The weight in gm. of 1 ml. of

blood, which is the density, is the weight of the plasma plus the weight of the cells in 1 ml. of blood.

$$(1) D_B = D_C V_C + D_P (1 - V_C)$$

Since G_B , G_C , and G_P all are the corresponding D values multiplied by the same factor (1.003 when $t = 25^{\circ}$) the gravity (D_t^t) values may be substituted for densities D_4^t in all terms of Equation 1.

(2)
$$G_B = G_C V_C + G_P (1 - V_C)$$

whence

$$V_C = \frac{G_B - G_P}{G_C - G_P}$$

Blood Hb concentration is cell volume × cell Hb concentration.

(4)
$$Hb_B = Hb_C V_C = Hb_C \times \frac{G_B - G_P}{G_C - G_P}$$

Constants must be substituted for Hb_c and G_c . Hb_c is calculated as Hb_B/V_c , with Hb_B determined by chemical analysis and V_c by hematocrit under standard conditions. G_c is calculated by rearrangement of Equation 2.

$$G_C = G_P + \frac{G_B - G_P}{V_C}$$

The V_c for Equation 5 is determined by hematocrit.

From data given in the experimental part, Hb_c for normal human blood cells is taken as 33.9 gm. per 100 ml., and the corresponding G_c as 1.0964. We have taken these as the constants. Substituting them for Hb_c and G_c in Equation 4 gives

(6)
$$Hb_B = 33.9 \times \frac{G_B - G_P}{1.0964 - G_P}$$

Cause of Relative Non-Effect of Cell Abnormalities on Calculation of Hemoglobin by Equation 6

As mentioned above, in blood with cells of abnormally low density deviations of Hb_c from 33.9 and of G_c from 1.0964 parallel each other in such a manner that the changes nearly cancel each other, numerator and denominator of the fraction $(33.9(G_B - G_P))/(1.0964 - G_P)$ being changed in nearly the same proportion by parallel deviations of the two constants.

In the hypochromic bloods of Table II, in fact, the correlation between abnormally low G_c and the error in gravity-calculated Hb_B is so slight that

it is not statistically definite, although in several bloods G_{σ} is below 1.088 and Hb_{σ} below 29 gm. per 100 ml.

TABLE I

Data from Twenty Normal Men

		Directly m	easured		Calculated					
			Hemat-		Cel	la .	Whole	blood		
Subject No.	GB, whole	GP, plasma	ocrit, centri- fuged	HbO ₂ , blood Hb by	Cala	1 5 E	Hb from GB and GP			
	gravity	gravity	cells per 100 ml. blood		G _C by Equation 5	Cell Hb = 100 HbO; Hematocrit	By Equation 6	Deviation from HbOs		
	D装	D 25	ml.	gm. per 100 ml.	D装	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.		
1	1.0635	1.0275	51.2	17.56	1.0978	34.3	17.66	+0.10		
2	625	262	48.0	17.46	1018	36.4	17.53	+0.07		
3	618	265	49.0	17.10	985	35.6	17.12	+0.02		
4	605	277	48.4	16.46	955	34.0	16.18	-0.28		
5	615	287	48.0	16.35	970	34.0	16.42	+0.07		
6	602	275	47.0	15.99	971	34.0	16.09	+0.10		
7	592	265	48.0	15.99	946	33.3	15.86	-0.13		
8	585	263	46.2	15.92	960	34.5	15.58	-0.34		
9	595	270	48.0	15.84	949	33.0	15.88	+0.04		
10	597	285	47.0	15.82	949	33.7	15.58	-0.24		
11	. 590	265	46.0	15.78	971	34.3	15.76	-0.02		
12	588	263	47.3	15.68	950	33.2	15.72	+0.04		
13	595	277	48.0	15.63	939	32.5	15.69	+0.06		
14	582	267	46.0	15.47	952	33.6	15.32	-0.15		
15	588	265	46.4	15.46	961	33.3	15.66	+0.20		
16	587	277	45.0	15.12	966	33.6	15.30	+0.18		
17	582	273	44.0	15.12	976	34.3	15.16	+0.04		
18	568	250	44.3	15.11	968	34.1	15.10	-0.01		
19	570	257	45.0	15.03	952	33.4	15.01	-0.02		
20	577	267	45.0	15.01	956	33.3	15.08	+0.07		
Mean	1.0595	1.0269	46.9	15.90	1.0964	33.9	15.89	-0.01		
s.D. from mean	± 0.0021	± 0.0009	± 1.8	± 1.00	± 0.0018			± 0.15		
Maximum + devia-								+0.20		
tion from HbO2										
Maximum minus								-0.34		
deviation from										
HbO_{2}										

Estimation of Hemoglobin from Specific Gravity of Whole Blood Alone In blood with plasma gravities within the normal range 1.027 ± 0.002 (Table I) one can, without much increase in error, estimate Hb_B from G_B

VAN SLYKE, PHILLIPS, DOLE, HAMILTON, ARCHIBALD, AND PLAZIN

TABLE II

Data from Women Rejected As Blood Donors, Apparently Healthy But with

Subnormal Hemoglobin

	1	Directly :	measured	l	Calculated					
			Hemat-		Ce	lis	Whole	blood		
Subject No.	GB, blood specific	GP, plasma	a fuged	HbCO, blood Hb by CO	G c by	Scrit Co.	Hb from Gs and GP			
	gravity	specific gravity	cells per 100 ml. blood	ca- pacity	Equa- tion 5	Cell Hb = 100 HbC0 Hematocrit	By Equa- tion 6	Devia- tion from HbCO		
	1D 35	D 25	ml.	gm. per 100 ml.	D 25	ĝm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.		
1	1.0525	1.0255	39.9	12.96	1.0932	32.5	12.91	-0.05		
2	510	265	37.6	11.65	917	31.0	11.88	+0.23		
3	506	275	37.5	11.50	891	30.7	11.37	-0.13		
4	503	255	37.0	11.79	925	31.9	11.86	+0.07		
5	508	275	37.0	11.76	895	31.8	11.46	-0.30		
6	528	265	1	12.48	944	1	12.75	+0.27		
7	528	275		12.50	908	1	12.45	-0.05		
8	542	290	42.7	12.28	880	28.8	12.68	+0.40		
9	480	260	36.1	10.24	869	28.4	10.59	+0.25		
10	504	270	41.2	11.64	838	28.2	11.43	-0.21		
11	500			11.65	940		12.02	+0.37		
12	545	270	41.5	12.96	933	31.2	13.43	+0.47		
13	522	1		12.88	869		12.62	-0.26		
14	520	1	1	12.63	944		12.67	+0.04		
15	508	250	38.4	12.54	920	32.6	12.22	-0.32		
16	524	275	39.7	12.25	904	30.8	12.25	+0.00		
17	500	265	37.7	11.13	889	29.6	11.40	+0.27		
Mean	1.0515	1.0265	39.0	12.05	1.0906	30.9	12.12	+0.08		
s.p. from mean								±0.26		
Maximum + deviation from HbCO								+0.47		
Maximum minus devi- ation from HbCO								-0.32		

alone, assuming that the plasma has the mean normal gravity, $G_{\mathbb{P}}$ 1.0269. Equation 6 then simplifies to

(7)
$$Hb_B = 480(G_B - 1.0269)$$

The maximal error in such bloods, introduced by a deviation of ± 0.002 from the assumed G_P of 1.0269, is about ± 0.5 gm. of Hb per 100 ml. of blood, or 3 per cent of normal Hb_B. The data in Table IV indicate that

the observed error is of this order in normal bloods, and in simple anemia. due to iron deficit or blood loss, of the type of the blood donors in Table II. When varied pathological conditions cause wide fluctuations in plasma protein concentration and G_P , however (last column, Table IV), greater errors can arise in Hb_B estimations made from G_B alone. Each gm. per 100 ml. of plasma by which the plasma proteins deviate above or below 7.4 gm. per 100 ml. causes a plus error in the same direction of about 0.7 gm. per 100 ml. of blood in the hemoglobin estimated by Equation 7. The latter can be used to follow hemoglobin changes in subjects that have been found to have plasma gravities within the range 1.025 to 1.029, and such use permits following Hb changes with finger blood. The extent of error that could arise, however, from undetected abnormality in plasma gravity is shown by a case with 12.7 gm. of plasma protein per 100 ml. reported by Atchley, Bacon, Curran, and David (3), in which the Hb_B calculated by Equation 7 was 4.7 gm. per 100 ml. of blood too high. Forty-nine other cases reported by Atchley et al. (3) from a general medical ward showed a distribution of error (estimated as the deviation of Hb_B by Equation 7 from Hb_B by Equation 6) similar to that of the last column of Table IV, 94 per cent of the cases showing an error of less than 1.5 gm. of Hb per 100 ml.

Calculation of Hematocrit Values from G_B and G_P

In Ashworth's (1, 4) papers, and in preliminary reports by the authors (5), Equation 3 with 1.097 as constant value of G_C was given as a means for approximate calculation of $V_{\mathcal{C}}$ from $G_{\mathcal{B}}$ and $G_{\mathcal{P}}$, although it was recognized that the gravity-calculated V_c showed, from hematocrit-determined V_c, percentage deviations that were much greater, especially in pathological blood, than the deviations between gravity-calculated and chemically determined Hb_B values. The reason for such V_c deviation is obvious from comparison of Equations 3 and 6. If the cell gravity deviates from the assumed 1.0964, its effect on gravity-calculated V_c is proportional to the change in the factor $1/(G_G - G_P)$, and is not canceled, as in Equation 6, by parallel change of a factor in the numerator. For example, in the blood of lowest G_c in Table II, Subject 10, the gravity-calculated Hb_B agrees with that gasometrically determined within the limits of error, while the V_c value calculated by Equation 3 is 0.339, 18 per cent below 0.412 obtained by hematocrit. In normal blood V_c can be calculated by Equation 3 within ± 0.02 (2 ml. of cells per 100 ml. of blood) of the hematocrit-determined value, a ±5 per cent maximal error, but the error increases so greatly, even in the moderately abnormal blood of subjects of the type in Table II, that calculation of V_c from gravities cannot be recommended, except in blood with normal cells.

EXPERIMENTAL

Blood was drawn with syringes from the arm vein and transferred to tubes containing 0.2 mg. of heparin per ml. of blood.

The volume of packed cells per 100 ml. (100 V_c) was determined by the conventional technique for clinical hematocrit values by use of Wintrobe (6) tubes. The blood was centrifuged at 3000 R.P.M. for 60 minutes, the center of each centrifuge tube being 18 cm. from the axis. (When a centrifuge was tried in which the centers of the tubes were only 9 cm. from the axis, 3000 R.P.M. for an hour gave with normal blood 51 ml. of cells per 100 ml. of blood, while the centrifuge with an 18 cm. axis gave 47.) The cell column obtained by the conventional technique contains, according to Gregersen and Schiro (7), about 7 per cent of its volume of inseparable plasma. The true cell volume would therefore be somewhat lower than the measured volume, and the true normal mean cell gravity somewhat higher than the 1.0964 obtained in Table I. The cell gravities, G_C, calculated in Tables I, II, and III, are the gravities of the packed red cells plus adherent plasma. If, applying Equation 5 to the mean normal values of Table I, one decreases V_c by 7 per cent, the value obtained for average normal G_c becomes 1.1013 instead of 1.0964, and the Hb concentration of the cells would be 36.3 instead of 33.9 gm. per 100 ml.

Blood hemoglobin concentrations were determined gasometrically. hemoglobin values of Table I were obtained by a modification of Van Slyke and Neill's (8) oxygen capacity method, with details to obtain maximal accuracy. Of each blood, soon after drawing, a portion of about 10 ml. was placed in a separatory funnel of 250 ml. capacity, and aerated by rotation as described by Stadie (9). After about 20 minutes 1 ml. was transferred to the Van Slyke-Neill manometric apparatus and the O₂ + CO content was determined. Both gases, mixed with the CO₂ and N₂, were set free from the blood by ferricyanide, the CO2 was absorbed, and the residual O₂ + CO + N₂ was measured. From the gas thus measured the sum of O₂ + N₂ physically dissolved in blood saturated with air at the observed temperature and barometric pressure was estimated from the N₂ and O₂ solubilities in blood ((10, 11); see also Fig. 48 (12)) in order to obtain the O₂ + CO bound by the hemoglobin. This procedure was used instead of determination of the O₂ alone by absorption from the extracted gases with Na₂S₂O₄ solution, because the latter procedure fails to include the small amounts of CO, in the neighborhood of 0.3 volume per cent, bound as HbCO that appear to be present in normal blood regularly, at least in New York. This normal blood CO is increased by smoking, which may raise it to as high as 1 volume per cent. Only a small part of such CO is displaced by O2 in the process of aerating. The mean oxygen capacity yielded by our results, 21.6 volumes per cent, is higher than the 20.7 previously estimated from oxygen capacity determinations ((12) p. 662) from normal men.

The determinations were done in duplicate or triplicate. The aeration in the Stadie rotator was continued during the entire period of the analyses, usually 40 to 60 minutes, so that any changes that might occur, such as formation of methemoglobin or transformation of methemoglobin present in vivo to active hemoglobin in vitro, might be detected. The accepted results were those in which consecutive analyses gave oxygen capacities within 0.05 volume per cent of each other. It has been shown that during the prolonged aeration any methemoglobin present is changed to active hemoglobin to such an extent that the oxygen capacity is 99 to 100 per cent of the total hemoglobin (13) in normal blood.

The hemoglobin values in Tables II and III were obtained by the "total hemoglobin" method of Van Slyke et al. (13); Na₂S₂O₄ is added to reduce any methemoglobin present and the carbon monoxide-binding capacity of the Hb is determined as a measure of the hemoglobin. The total hemoglobin is designated as HbCO in Tables II and III.

Specific gravities of whole blood and plasma were determined by the copper sulfate method previously described (2).

The values for cell gravities and cell hemoglobin concentration are calculated in Table I to obtain from normal blood the constants for Equation 6. In Tables II and III they are included to indicate the nature of cell abnormalities in the bloods examined. It will be noted that the group in Table II, although composed of women apparently healthy except for mild anemia, showed more marked tendency to low blood cell gravity and cell hemoglobin than did the patients of Table III. In Table IV are summarized the results which have already been discussed in connection with Equation 7.

Accuracy of Calculation of Blood Hemoglobin Concentration from Gravities of Whole Blood and Plasma

The results in Tables II and III indicate that, in patients with various types of anemia, the maximal deviation of Hb, estimated from gravities measured by the copper sulfate method, from Hb gasometrically determined, is about ± 0.6 gm. per 100 ml. of blood; this figure is indicated both by the maximal observed deviations and by estimation as twice the standard deviation. If 0.1 gm. per 100 ml. is allowed as the error of the gasometric determination, the maximal error of the gravity-determined Hb in pathological bloods of the types studied may be estimated as about ± 0.5 gm. per 100 ml., or 3 per cent of the normal hemoglobin concentration.

VAN SLYKE, PHILLIPS, DOLE, HAMILTON, ARCHIBALD, 357 AND PLAZIN

TABLE III Blood from Miscellaneous Hospital Patients on Medical Service

***************************************		Directly	measured		Calculated					
			Hemato-		Ce	lls	Whole	e blood		
Patient No.	crit, cen-	HbCO, blood Hb by CO	Gc by	HbCO atocrit	Hb from (GB and GP				
			100 ml. blood	capacity	Equation 5	Cell Hb 100 HbCO Hematocrit	By Equation 6	Deviation from HbCO		
	D 25	D 25	ml.	gm. per 100 ml.	D 25	gm. per 100 ml.	gm. per 100 ml,	gm. per 100 ml.		
1*	1.0448	1.0285	24.0	8.25	1.0964	34.4	8.02	-0.23		
2	440	273	24.2	8.43	963	34.8	8.19	-0.24		
3*	468	268	28.9	9.50	960	32.9	9.74	+0.24		
4	495	290	31.0	10.32	951	34.6	10.31	-0.01		
5	448	249	28.0	9.59	960	34.2	9.43	-0.16		
6†	289	225	9.2	3.28	920	35.7	2.94	-0.34		
7‡	589	313	42.8	14.17	958	33.1	14.37	+0.20		
8	389	243	20.2	6.93	965	34.3	6.86	-0.07		
9	485	249	33.2	11.03	960	33.2	11.18	+0.15		
10	465	250	30.4	9.86	957	32.4	10.21	+0.35		
11	520	268	36.3	12.81	962	35.3	12.28	-0.53		
12	501	255	35.0	11.85	958	33.8	11.76	-0.09		
13	485	290	29.2	9.90	958	33.9	9.82	-0.08		
14	466	228	32.8	11.48	954	35.0	10.96	-0.52		
15§	543	295	37.5	12.45	956	33.2	12.57	+0.12		
16‡	640	336	48.6	16.31	961	33.6	16.41	+0.11		
17	623	292	49.6	16.50	959	33.3	16.70	+0.20		
18	545	259	40.9	13.85	958	33.9	13.76	-0.11		
19	565	291	41.0	13.56	959	33.1	13.81	+0.25		
20	585	290	44.2	14.96	957	33.8	14.82	-0.14		
21	425	253	24.5	7.94	955	32.4	8.20	+0.26		
22	531	290	36.0	12.25	959	34.0	12.12	-0.13		
23	482	267	31.2	10.66	960	34.2	10.52	-0.14		
24	470	244	32.0	11.05	950	34.5	10.64	-0.41		
25	532	290	36.4	12.34	955	33.9	12.17	-0.17		
26	515	279	34.9	11.70	955	33.5	11.68	-0.02		
27	555	270	41.6	13.75	955	33.1	13.92	+0.17		
28	531	247	40.0	13.69	957	34.2	13.43	-0.26		
29	511	260	36.1	12.13	955	33.6	12.09	-0.04		
30	465	273	28.4	9.66	949	34.0	9.42	-0.24		

TABLE	TTI	Con	c711	hah

Directly measured						Calculated					
		Hemato-	Vernata		lls	Whole blood					
Patient No.	G _B		crit, cen- trifuged	HbCO, blood Hb by CO	Gc	HbCO atocrit	Hb from (GB and GP			
	100 ml. capacity	100 ml. capacity Form					Cell Hb 100 HbCO		Deviation from HbCO		
	D 25 35	D 25	ml.	gm. per 100 ml.	D 25	gm. per 100 ml.	gm. per 100 ml	gm. per 100 ml.			
31¶	570	262	44.2	14.77	952	33.3	14.74	-0.03			
32¶	585	26 8	45.9	15.29	959	33.3	15.45	+0.16			
33¶	572	260	44.9	14.97	955	33.2	15.02	+0.05			
34¶	545	266	40.1	13.41	962	33.4	13.55	+0.14			
35¶	554	260	42.1	13.64	959	32.2	14 15	+0.51			
36¶	571	260	44.5	14.87	958	33.4	14 95	+0.08			
37	473	297	26.6	9.00	959	33.8	8 95	-0.05			
38	428	280	22.0	7.42	952	33.7	7.34	-0.08			
3 9	421	242	25.1	7.93	954	31 6	8.41	+0.48			
40	470	271	29.0	9.97	957	34.4	9.73	-0.24			
41	526	290	35.2	11.64	960	33.1	11 87	+0.23			
42§	485	274	31.1	10.41	952	33.3	10.36	-0.05			
43§	389	273	17.0	6.41	956	37.7	5 69	-0.72			
44	562	269	42.2	13.96	963	33.1	14 29	+0.33			
45	569	266	43.7	14.79	960	33 9	14.71	-0.08			
46	509	252	36.3	12 37	960	33.9	12 24	-0.13			
47	473	258	3 0 9	9.81	953	31 7	10 32	+0.51			
Mean devia	tion from	НьСО	· · · · · · · · · · · · · · · · · · ·			<u> </u>		-0.02			
s.d. from n	nean devia	ation		•				±0.26			
Maximum -	+deviatio	n from I	HbCO				•	+0.51			
" m	inus devi	ation fro	om HbC0)				-0.72			

^{*} Pernicious anemia.

The error is about the same in blood with low as in blood with high hemoglobin concentration; it is an error constant in gm. per 100 ml. rather than in percentage of the hemoglobin present.

An important part of the error in hemoglobin calculated by Equation 6 from gravities in Tables I, II, and III is attributable to the limit of error of

[†] Advanced nephritis; deficit of both hemoglobin and plasma protein.

[‡] Myeloma; high plasma proteins.

[§] Hodgkin's disease.

^{||} Cirrhosis of the liver.

[¶] Hepatitis.

the gravity measurements by the copper sulfate method. The greatest error in Hb occurs when errors in G_B and G_P occur in opposite directions. If a plus-minus error in G_B exceeding the standard error occurs once in 3 times, a plus error equal to the standard error occurs once in 6 times. Similarly a minus error in G_P equal to the standard error occurs once in 6 times. Simultaneous occurrence of the plus error in G_B and the minus error in G_P would occur once in 36 times, and the reverse combination also occurs once in 36, so that errors in Hb from gravity errors exceeding their standard deviations, and in opposite directions, would occur once in 18 times. If the "maximal" error is taken as one that would be exceeded once in 20 times, the maximal error of Hb calculated by Equation 6 would therefore approx-

Table IV

Error of Hb Calculated from G_B Alone As Hb = $480(G_B - 1.0269)$

ource of data ype of subjects. Cotal No. of subjects.				Norm	I d men	Table II Rejected women blood donors	Table III Hospital patients		
							cent	per coni	per cont
Subjects	with	error	under	0.5	gm.	90	(18)*	94 (16)	62 (29)
"	44	"	"	1.0	-	1	(20)	100 (17)	81 (39)
46	44	"	"	1.5	"		• /		92 (44)
u	"	"	"	2.0	44				100 (47)
						gm. 10	IIb per	gm. Hb per 100 ml.	gm. Hb per 100 ml.
Mean error						+0	.03	-0.01	+0.05
s.v. of error f	rom 1	nean .				±0	. 27	±0.28	±0.76

^{*} The figures in parentheses denote the number of subjects.

imate (slightly exceed) the summated effect of opposite errors in G_P and G_B equal to their standard deviations, which have been found to be ± 0.0003 and ± 0.0004 gravity unit respectively (2). The error in Hb_B caused by errors of +0.0004 in G_B and -0.0003 in G_P is approximately 0.3 gm. of Hb per 100 ml. of blood, which is about the maximal error found in gravity analyses of normal blood (Table I). It appears that nearly all of the error of Hb_B calculated from G_B and G_P in normal subjects (Table I) is attributable to the error of the copper sulfate gravity method, and about half the error in the abnormal bloods (Tables II and III). A gravity method free

¹Standard error = standard deviation of gravity by CuSO₄ method from gravity by pycnometer. By the probability equation, 0.317, or 1/3.15, of the gravities by CuSO₄ may be expected to deviate from pycnometric gravities by more than the standard error.

of error could be expected to reduce the "maximal" Hb error in normal blood to nearly zero, and in pathological blood to 0.2 or 0.3 gm. per 100 ml.

In Fig. 1, for convenience in rapid calculation, is a nomogram prepared by standard methods (14) for calculation of plasma protein and blood hemoglobin concentrations from gravities. The plasma values are calculated by Equation 3 of the preceding paper (15), the hematocrit and hemoglobin values by Equations 3 and 6 respectively of this paper.

SUMMARY

In twenty normal and 64 abnormal, mostly anemic, heparinized human bloods, specific gravities were determined by the copper sulfate method. From specific gravities of the whole blood and plasma, blood hemoglobin concentrations were calculated by the formula of Ashworth and Tigertt, and were compared with hemoglobin concentrations determined by precise gasometric methods.

The standard deviation of the gravity-determined from gasometric hemoglobin was ± 0.15 gm. per 100 ml. of blood in the normal bloods, and ± 0.26 gm. in the abnormal.

A portion of the deviation equal to nearly all that observed in the normal bloods is estimated to be attributable to the limit of accuracy of the copper sulfate gravity method.

A nomogram is presented for rapid calculation of plasma protein and blood hemoglobin concentrations from specific gravities.

BIBLIOGRAPHY

- 1. Ashworth, C. T., and Tigertt, W. D., J. Lab. and Clin. Med., 26, 1545 (1940-41).
- Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., J. Biol. Chem., 183, 305 (1950).
- Atchley, J., Bacon, R., Curran, G., and David, K., J. Lab. and Clin. Med., 30, 830 (1945).
- 4. Ashworth, C. T., and Adams, G., J. Lab. and Clin. Med., 26, 1934 (1940-41).
- Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., Bur. Med. News Letter U. S. Navy, 1, No. 9, 1 (1943).
- 6. Wintrobe, M. M., J. Lab. and Clin. Med., 15, 287 (1929).
- 7. Gregersen, M. I., and Schiro, H., Am. J. Physiol., 121, 284 (1938).
- 8. Van Slyke, D. D., and Neill, J. M., J. Biol. Chem., 61, 523 (1924).
- 9. Stadie, W. C., J. Biol. Chem., 49, 43 (1921).
- 10. Van Slyke, D. D., Dillon, R. T., and Margaria, R., J. Biol. Chem., 105, 571 (1934).
- Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., J. Biol. Chem., 105, 597 (1934).
- Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore (1943).
- Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O., J. Biol. Chem., 166, 121 (1946).
- 14. Allcock, H. J., and Jones, J. R., The nomogram, London (1932).
- 15. Van Slyke, D. D., Hiller, A., Phillips, R. A., Hamilton, P. B., Dole, V. P., Archibald, R. M., and Eder, H. A., *J. Biol. Chem.*, **183**, 331 (1950).

THE ACTIVITY OF CERTAIN SUBSTITUTED INDOLEACETIC ACIDS AS PLANT HORMONES IN THE PEA TEST

BY STEPHEN P. FINDLAY AND GREGG DOUGHERTY

(From the Frick Chemical Laboratory, Princeton University, Princeton)

(Received for publication, July 1, 1949)

Although Went and his associates imply that the introduction of a methoxy group to an indolealkanoic acid effects its inactivation as a growth factor in the pea test for plant hormones (1, 2), this phenomenon has been observed only in the case of the 5-, 6-, and 7-methoxy derivatives of indolepropionic acid (2). Therefore, it seemed worth while to examine. at least qualitatively, the behavior in this test of the homologous 5-, 6-, and 7-methoxyindoleacetic acids, the synthesis of which has been described elsewhere (3). It has been found that treatment of etiolated pea stems, prepared for experiments essentially as described by van Overbeek and Went (4), with solutions of these substituted indoleacetic acids causes unmistakable inward curvatures characteristic of stem growth. Such curvatures are thought to ensue because under the experimental conditions the inner, cut surface has become insensitive to auxin-like substances; thus when the cut stems are immersed in solutions of such compounds only the outer, intact surface responds and the resulting growth differential is manifest as an inward curvature (5). The curvatures observed were of approximately the same degree as those obtained with indoleacetic acid. This is apparent from Fig. 1 where the stems treated with 5- and 6-methoxy derivatives are compared with those treated with indoleacetic acid itself and with tap water. One concludes that the inactivation produced by the introduction of a methoxy group may be peculiar to indolepropionic acid.

It is noteworthy also that 1- and 3-naphthazoleacetic acids (Fig. 2) cause growth curvatures in etiolated pea stems. However, Δ' -cyclopenteneacetic acid which satisfies all of Went's structural requirements for activity in this test (2) is apparently inert.

EXPERIMENTAL

Etiolated Pea Seedlings—Alaska peas (kindly provided by the Bristol Seed Company), after being soaked for 6 hours in tap water, were planted in ice box dishes each containing 5 pounds of washed sea sand moistened with 400 ml. of water. The dishes, each containing twenty-eight seeds, were placed under boxes in a closet until the seedlings had attained a length of 12 to 15 cm. This required 6 days in midsummer.

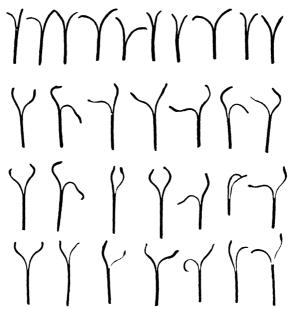


Fig. 1. Top row, etiolated pea stems after longitudinal splitting and several hours immersion in water (no apparent inward curvature). Second row, etiolated pea stems after several hours in 10^{-5} m 3-indoleacetic acid, the control. Third row, stems after similar interval in 10^{-5} m 5-methoxy-3-indoleacetic acid. Bottom row, stems after immersion in 10^{-5} m 6-methoxy-3-indoleacetic acid.

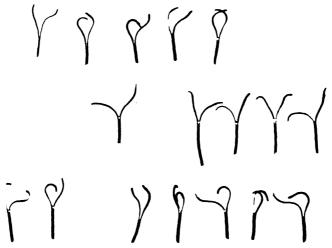


Fig. 72. 7Top row, etiolated pea stems after several hours immersion in 10⁻⁵ m 3-indoleacetic acid, the control. Middle row, stems after immersion in 10⁻⁵ m 3-naphthazoleacetic acid. Bottom row, stems after immersion in 10⁻⁵ m 1-naphthazoleacetic acid.

Preparation and Treatment of Stems—The seedlings were prepared for testing approximately as described by van Overbeek and Went (4) (cf. Figs. 1 and 2). After being washed for 2 hours in tap water in the dark, the stems were immersed 8 to 16 hours in 25 ml. volumes of test solution contained in Petri dishes, five to eight stems to a dish.

In each experiment, two compounds were examined for activity in comparison with indoleacetic acid as a control, and for each of the three substances dilutions of 10⁻⁵ and 10⁻⁶ m were used.

Preparation of Test Solutions—Stock solutions were prepared before each experiment by dissolving 1.0×10^{-4} mole of the control or test substances

TABLE I
Response of Pea Seedling Stems to Auxin-Like Substances

Compound	Concentra- tion of solution	No. of stems	No. of stems responding positively
	¥		
3-Indôleacetic acid (control)	10-6	49	49
	10-4	36	36
5-Methoxy-3-indoleacetic acid	10-4	19	17
" "	10-6	11	11
6-Methoxy-3-indoleacetic "	10-	7	7
" "	10-6	2	2
7-Methoxy-3-indoleacetic "	10-6	20	12
" "	10-6	8	0
1-Naphthazoleacetic acid	10-6	16	16
" "	10-6	14	14
3-Naphthazoleacetic "	10-6	14	6
•	10⁻●	9	0
Cyclopenteneacetic acid	10-1	14	0
<u>"</u> """	10-6	14	0

in 0.84 ml. of 0.120 N sodium hydroxide and 0.5 ml. of 95 per cent ethanol and diluting to 100 ml. The stock solution (1.0 ml.) diluted to 100 ml. provided the 10^{-5} M dilution and the appropriate treatment of this furnished the 10^{-6} M dilution.

Recording of Results—The stems, after overnight immersion in the solutions of auxin-like compounds, were freed of excess water, examined for response, and, when desirable, printed on high contrast, glossy surface Kodabromide paper (see Table I and Figs. 1 and 2).

SUMMARY

It has been found that, unlike the analogous derivatives of indolepropionic acid, 5-, 6-, and 7-methoxyindoleacetic acids act as plant hormones in the pea test. Also, 1- and 3-naphthazoleacetic acids are active in this test, but Δ' -cyclopenteneacetic acid is not.

BIBLIOGRAPHY

- 1. Went, F. W., and Thimann, K. V., Phytohormones, New York, 115 (1937).
- Koepfli, J. B., Thimann, K. V., and Went, F. W., J. Biol. Chem., 122, 763 (1937–38).
- 3. Findlay, S. P., and Dougherty, G., J. Org. Chem., 13, 560 (1948).
- 4. van Overbeek, J., and Went, F. W., Bot. Gaz., 99, 22 (1937).
- 5. Went, F. W., Bull. Torrey Bot. Club, 66, 391 (1939).

STUDIES ON THE EXCRETION OF ADRENOCORTICAL COMPOUNDS

I. ISOLATION OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE AND OTHER COMPOUNDS FROM THE URINE OF NORMAL MALES*

By JOHN J. SCHNEIDER

(From the Department of Experimental Medicine, Jefferson Medical College, Philadelphia)

(Received for publication, October 20, 1949)

Although it can be said with reasonable certainty that the adrenocortical compounds elaborated by man will prove to be qualitatively similar to those already isolated from the adrenal glands of animals, it would be desirable to obtain evidence of a more direct sort. Necessarily, the approach to this problem has been indirect and has largely centered about the isolation and identification of a number of metabolic (degradation) products excreted in the urine. These compounds include both a wide variety of C-19 17-ketosteroids and a small number of C-21 compounds, which have been generally and in some instances closely associated with adrenocortical function (see Engstrom (2); Dorfman (3); Heard (4)).

Urine normally contains, in addition, what appears to be a very different group of substances, all included in what is usually termed the "cortin" fraction. Although the identity of the substances comprising this fraction remains unknown, both its chemical behavior and biological activity (see Heard (4)) suggest that it consists of a mixture of compounds of the adrenocortical type which have "escaped" metabolic degradation. This view was in part substantiated when Venning, Hoffman, and Browne (5) isolated a crystalline ketone, m.p. 234–236°, from postoperative urine which reduced alkaline silver and showed biological activity (promotion of glycogen storage in the liver). More recently, evidence of another sort was added when Mason and Sprague (6) described the recovery of appreciable amounts of 17-hydroxycorticosterone from the urine of a patient who had Cushing's syndrome and associated severe diabetes.

It is the purpose of this communication to describe a procedure for the isolation of compounds of the adrenocortical type from human urinary sources. The method includes extraction with chloroform, followed by recovery of the ketonic fraction, its partitioning between benzene and water, and chromatography of the acetylated residues. By this procedure it has been possible to isolate five crystalline compounds of the adrenocortical type and a sixth in a non-crystalline state.

^{*}A preliminary announcement has appeared (1).

Procedures and Methods

Collection and Extraction—Urine was collected daily over chloroform in amounts of from 20 to 30 liters from a group of male medical students.

Prior to any adjustment of pH the fresh, pooled urine was extracted by shaking three to four times with 0.1 volume of chloroform¹ to give a free (i.e. unconjugated) fraction. The pale yellow extracts were combined and concentrated to a small volume at low temperature in vacuo, and the concentrates so obtained stored as such in the cold. In some instances the urine was stored over chloroform in the cold for periods up to 12 hours and then extracted as indicated above.

After the removal of the free fraction, the pH of the urine was adjusted to 1 by the addition of concentrated hydrochloric acid, and over the course of the next 3 hours it was reextracted three to four times with chloroform. These extracts were combined and treated in the manner employed for the free fraction. The crude dark brown material so obtained was designated Fraction C-1 and was assumed to consist largely of those originally conjugated compounds relatively easily hydrolyzed by hydrochloric acid. Finally, the acidified urine was reextracted three to four times after standing at pH 1 an additional 24 hours. The dark red extract obtained in this case was assumed to consist of those substances relatively slowly liberated from the conjugated form by hydrochloric acid and was designated Fraction C-2.

At weekly intervals the accumulated chloroform concentrates were appropriately pooled, brought to a volume of about 2000 ml., and while still cold washed twice with 0.2 volume of cold 0.1 n NaOH and twice with distilled water. The extracts were then carefully reduced to dryness at low temperature *in vacuo* and stored in the cold under nitrogen. At the end of the collection period similar dried residues were combined to give a light brown, largely crystalline free fraction, a red-brown semicrystalline Fraction C-1, and a dark red, resinous Fraction C-2. By this procedure a total of 1000 liters of urine was extracted over a period of 10 weeks.

70 Per Cent Alcohol-Petroleum Ether Fractionation—As an initial step, designed both to reduce the weight of the crude fractions and in part to eliminate emulsion-stabilizing substances, the crude extracts were divided into 70 per cent alcohol-soluble and petroleum ether-soluble parts. This procedure was carried out by taking up the crude material in from 5 to 10 volumes of absolute alcohol, adding an equal volume of petroleum ether, and shaking and separating the layers after the addition of sufficient water to adjust the alcohol to 70 per cent (by volume) concentration.

¹ Merck's U. S. P. grade, distilled after standing over anhydrous potassium carbonate.

The alcoholic phase was reextracted three times with 0.3 volume of petroleum ether and the petroleum ether phases combined and extracted twice with 0.2 volume of 70 per cent alcohol. The respective solutions were taken to dryness *in vacuo* at low temperature.

Benzene-Water Partition and Girard Separation—The procedure of benzene-water partitioning employed is based on the method described by Mason, Myers, and Kendall (7). In the case of adrenal extracts, in which the ratio of ketonic to non-ketonic elements is relatively high, these authors demonstrated that the procedure could be effectively applied to relatively crude fractions. The same procedure applied as such to crude urinary extracts, in which the ketonic-non-ketonic ratio is very low, is ineffective and becomes useful only when the non-ketonic elements (an important member being caffeine²) are removed. In these initial experiments, the over-all procedure consisted of dividing the 70 per cent alcohol-soluble fraction into benzene-soluble and water-soluble parts, followed by recovery of the ketonic moieties of each and their extensive partitioning between benzene and water.³

In each case the initial division into benzene-soluble and water-soluble components was carried out by taking up the crude 70 per cent alcohol-soluble material in approximately 100 ml. of benzene, and extracting this fifteen times with equal volumes of distilled water. Both phases were reduced to dryness in vacuo. The water residue contained large amounts of caffeine, but was substantially lighter in color than the benzene residue.

The ketonic parts of both residues were then separately isolated with Girard's Reagent T under mild conditions. To do this each crude fraction was taken up in 10 to 20 ml. of a 50 per cent (by volume) solution of glacial acetic acid in 90 per cent methanol. An amount of Girard's reagent equal to 5 times the weight of the crude fraction was added and the whole warmed sufficiently to achieve solution. The mixture was then allowed to stand at room temperature for 12 to 18 hours, after which there were added ice, chloroform, and, with shaking, sufficient cold 5 N NaOH to neutralize the acetic acid. The non-ketonic elements were removed by extracting the mixture with eight 0.3 volumes of chloroform. At this point the pH of the still cold mixture was adjusted to 1 by the cautious addition of concentrated hydrochloric acid and the ketonic fraction removed by repeated extraction with chloroform, both immediately and at intervals up to 24 hours. In each instance the non-ketonic frac-

² Although a diketone, caffeine fails to react as such under the conditions of the Girard separation here employed and regularly appears in the non-ketonic fraction.

^{*} In future experiments it would appear more desirable to eliminate the initial division into benzene-soluble and water soluble parts and to partition the ketonic fraction directly.

tion was twice retreated with Girard's reagent to yield additional, small ketonic fractions which were combined with the original. The neutral ketonic fraction was ultimately obtained by washing the combined chloroform solutions with dilute, cold sodium carbonate solution and water and evaporating in vacuo.

In carrying out the benzene-water partitioning, the benzene-soluble and water-soluble ketonic fractions were separately taken up in 25 ml. of benzene and extracted with fifteen 25 ml. portions of distilled water. The benzene-soluble phases were combined and reduced to dryness to become the first benzene residue. The water phases were combined, concentrated to from 50 to 100 ml. at low temperature in vacuo, and in turn extracted fifteen times with equal volumes of benzene. The water phase was reduced to dryness to become the first water residue. The partitioning procedure was then continued in such a fashion as to yield ultimately three benzene residues, two water residues, and a third fraction (Fraction III of Mason, Myers, and Kendall (7)) which passed readily from water to benzene and from benzene to water, here designated the benzene-water fraction. In each instance the procedure served to concentrate the greater part of the colored materials in the benzene residues and to lighten the color of the benzene-water fraction progressively.

Chromatography—Prior to chromatography the various ketonic fractions were acetylated by allowing them to stand for 18 to 24 hours in a few ml. of a pyridine-acetic anhydride mixture. The greater part of the reagents was removed with a stream of nitrogen and the remainder by washing a chloroform solution of the residue with cold 2 n HCl, dilute cold sodium carbonate solution, and water.

A 1:1 mixture of magnesium silicate⁵ and Celite⁶ was used as the adsorbent in a column which was in most cases roughly 5 to 10 times as long as wide. The ratio of adsorbent mixture to acetylated ketonic fraction varied from 50:1 to 100:1. Solvents were forced through the column by positive (nitrogen) pressure. In most cases the chromatography procedure consisted of applying a benzene solution of the neutral acetylated mixture to the column prepared with petroleum ether, followed by development with petroleum ether, petroleum ether containing increasing amounts of benzene, benzene, benzene-ether mixtures, ether, and ether-ethyl acetate mixtures. A detailed account of the development of one chromatogram is given in Table I.

The petroleum ether used was Baker's c.p. grade and was further puri-

⁴ Too great concentration of the water phases is avoided, since this tends to promote the formation of emulsions in the course of the subsequent extraction with benzene.

⁶ Magnesium silicate No. 34, Philadelphia Quartz Company, Berkeley, California.

⁶ Analytical filter aid prepared by Johns-Manville.

fied by washing with concentrated sulfuric acid and water and was distilled after drying over sodium hydroxide flakes. The benzene was Merck's analytical reagent, thiophene-free grade, redistilled before use. Baker's c.p. absolute ether and ethyl acetate were used and, while not further dried, were redistilled before using.

TABLE I

Chromatogram 1, Acetylated Free Benzene-Water Residue (144 Mg.)

Magnesium silicate 3.6 gm., Celite 3.6 gm. (column, 15 × 145 mm.). Eluates removed and examined in 20 ml. volumes.

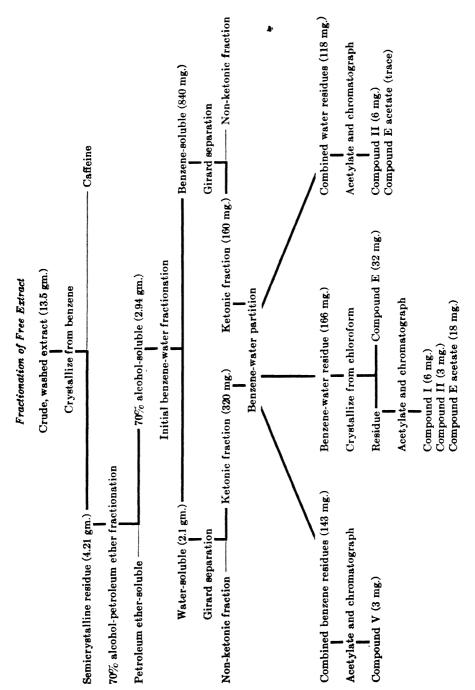
Composition of eluent	Volume	Eluate
· .	ml.	
Petroleum ether	250	
Benzene, 10-90% in petroleum		
ether	250 (Each)	0 to trace, non-crystalline
Benzene	250	0
Ether, 5% in benzene	500	0
" 10% " "	500	Trace, semicrystalline
		Crystalline, m.p. 226-228° (Compound I)
" 30% " "	500	Trace, non-crystalline
" 40% " "	500	" crystalline
" 50% " "	500	Crystalline, m.p. 234-238° (Compound VI)
" 60% " "	500	Crystalline, m.p. 234-238° (Compound VI)
" 70% " "	250	Trace, crystalline, m.p. 233-236° (Compound VI)
" 85% " "	250	Trace, non-crystalline
"		0
Ethyl acetate, 5% in ether	500	0
" " 10% " "	750	Crystalline, m.p. 263-266° (Compound II)
" " 20% " "	500	Trace, crystalline, m.p. 263-266° (Compound II)
" 40-100% in ether	250 (Each)	Non-crystalline

All melting points were determined on a Fisher-Johns type apparatus and are recorded as read. The absorption spectra were determined with a Beckman spectrophotometer.

Results

Fractionation of Free Fraction

The further treatment of the free fraction here detailed is given in outline form in the accompanying diagram. The same general procedure



was employed for Fractions C-1 and C-2, except that in these cases no attempt was made to remove the caffeine.

Isolation of Caffeine—The tan, semicrystalline, 13.5 gm. crude free fraction was taken up in benzene, concentrated to the point of crystallization, warmed, and allowed to stand at room temperature. The material which then separated was washed with cold ethanol and recrystallized from ethanol or benzene, taking the form of long, somewhat opaque needles,

TABLE II

Distribution of Crystalline Compounds in Chief Fractions

Fraction	Weight		Compounds isolated						
a paction	·	I	п	ш	IV*	v	VI		
	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
Free combined benzene residues	143					3			
" " water "	118		6				Trace		
" benzene-water residue	166	6	3	}			50†		
C-1, combined benzene residues	257								
" " water "	70			1					
" benzene-water residue	202		3	6			8		
C-2, combined benzene residues	343								
" " water "	53		l						
" benzene-water residue	245			3	4				

^{*} Non-crystalline.

m.p. 234-238°, with varying tendencies to sublime at lower temperatures. A small sample was sublimed for analysis.

Isolation of 17-Hydroxy-11-dehydrocorticosterone and Its Acetate (Compound VI, Table II)—Following the removal of the caffeine, the residue (4.21 gm.) was divided into petroleum ether-soluble and 70 per cent alcohol-soluble parts, and the latter (2.94 gm.) was redivided into a water-soluble portion (2.1 gm.) and a benzene-soluble portion (840 mg.). Treatment of both fractions with Girard's reagent gave water-soluble ketonic fractions totaling 320 mg. and benzene-soluble ketonic fractions totaling 160 mg. Both fractions were partitioned between benzene and water (by combining appropriate fractions) to yield three benzene residues totaling 143 mg., two water residues totaling 118 mg., and a single benzene-water residue of 166 mg.

In the course of reducing a chloroform solution of the free benzenewater residue to dryness, crystalline material separated which proved to

^{† 32} mg. as the free compound; 18 mg. as the acetate.

be free 17-hydroxy-11-dehydrocorticosterone (Kendall's Compound E, Reichstein's Substance Fa; (preliminary communication (1)). In addition to the 32 mg. recovered as the free steroid, 18 mg. of the corresponding acetate have since been isolated in the course of chromatographing the acetylated residues of the fraction it was obtained from. The compound was eluted upon the application of 40 to 70 per cent ether in benzene (by volume) solutions to a magnesium silicate-Celite column of the residue (Chromatogram 1), a detailed description of which is given in Table I.

The acetate crystallized from absolute ethanol in the form of needles, melted at 238–240° (after becoming opaque at around 200°), and showed no depression in melting point on admixture with an authentic preparation of 17-hydroxy-11-dehydrocorticosterone acetate. The addition of sulfuric acid to a small amount of the crystalline compound gave a yellow solution with a faint transient green fluorescence. Methanolic solutions of the acetate reduced ammoniacal silver at room temperature (somewhat more slowly than the free compound) and formed a red precipitate upon the addition of a few drops of a saturated solution of 2,4-dinitrophenylhydrazine in 2 n HCl (Brady's reagent).

Isolation of Compound I (Table II)—This compound was the first eluted from the column described in Table I. It was removed upon the application of a 10 to 20 per cent (by volume) solution of ether in benzene. Upon recrystallization from methanol it took the form of plates, m.p. 226-228°. Methanolic solutions reduced alkaline silver promptly and slowly gave a yellow-orange precipitate upon the addition of a few drops of Brady's reagent. When mixed with sulfuric acid, the crystals formed a yellow non-fluorescing solution.

Isolation of Compound II (Table II)—Small amounts of this compound were obtained from both the free benzene-water residue (Chromatogram 1, Table I) and the free water residues. In each case the compound was eluted upon the application of a 10 per cent (by volume) solution of ethyl acetate in ether and, when recrystallized from absolute ethanol, took the form of short needles. The melting point of the various fractions proved to be the same, $265-268^{\circ}$, with marked decomposition. Admixture of the crystalline substance with concentrated sulfuric acid generally gave light brown solutions. In methanolic solution the compound formed a redbrown and then a black precipitate when tested with alkaline silver and formed a red precipitate upon the addition of a few drops of Brady's reagent. The absorption spectrum as measured in the ultraviolet region was very similar to that obtained in the case of 17-hydroxy-11-dehydrocorticosterone, with a maximum at $237-238 \text{ m}\mu$.

Isolation of Compound V (Table II)—This substance, which has been obtained in very small amounts, is the only one as yet isolated from a benzene residue fraction. It was recovered in the course of chromato-

graphing the acetylated free combined benzene residues and was eluted upon the application of a 70 per cent (by volume) solution of benzene in petroleum ether. It was recrystallized from absolute ethanol to give short, fine needles, m.p. 228-230°. Like Compound I, it immediately reduced alkaline silver and gave an orange-yellow solution on treatment with Brady's reagent. On admixture with sulfuric acid it formed a yellow-brown solution.

Fractionation of Fractions C-1 and C-2

Isolation of 17-Hydroxy-11-dehydrocorticosterone Acetate (Compound VI, Table II)—An additional 8 mg. of 17-hydroxy-11-dehydrocorticosterone acetate were isolated in the course of chromatographing the acetylated benzene-water Fraction C-1. The properties of this sample were in every regard the same as those of the corresponding acetate isolated from the free fraction.

The over-all yield of 17-hydroxy-11-dehydrocorticosterone was thus raised to 55.3 mg., corresponding to 55.3 γ per liter.

Isolation of Compound III (Table II)—This substance was recovered in the course of chromatographing the benzene-water Fractions C-1 and C-2 and in each case was eluted upon the application of a 20 to 30 per cent (by volume) solution of ether in benzene. The crude material was readily recrystallized from absolute ethanol to give colorless, flat needles, m.p. 224-226°. Like Compounds I and V, this substance reduced ammoniacal silver in the cold (giving, however, first a brown, then a black precipitate) and gave a yellow-orange precipitate when treated with Brady's reagent. The melting point on admixture with Compound I (m.p. 227-228°) was from 222-226° to 224-226°. It differed from Compound I, however, in crystal form (needles rather than plates) and in the sulfuric acid test, the addition of sulfuric acid giving a brown to redbrown solution in contrast to the vellow solution characteristic of Compound I. On admixture with Reichstein's Substance V acetate (allopregnane- $3(\beta)$, $11(\beta)$, $17(\beta)$ -21-tetrol-20-one diacetate; needles, m.p. 223-224°) the melting point was 210-216°. While it is recognized that Compounds I and III may represent mixtures, they are tentatively regarded as different substances. Compound V is tentatively regarded as a distinct compound and different from Compounds I and III because it appeared in a different fraction and because it was eluted from the column well before the other two substances would be expected to appear.

Isolation of Compound II (Table II)—An additional 3 mg. of this compound, previously isolated from the free water residues and the free benzene-water fraction, were obtained when chromatographing the benzene-

⁷ The author wishes to thank Professor T. Reichstein, Pharmazeutische Anstalt der Universität, Basel, for generously supplying a sample of his Substance V acetate.

water Fraction C-1. It was eluted at the same point and was in all other regards identical with the acetate previously described.

Isolation of Compound IV (Table II)—This substance was isolated in a non-crystalline state in the course of chromatographing the acetylated benzene-water Fraction C-2. It was eluted with highly colored material upon washing the column with a 60 per cent (by volume) solution of benzene in petroleum ether. Methanolic solutions of the crude fraction immediately reduced alkaline silver and immediately formed a red precipitate upon the addition of Brady's reagent.

DISCUSSION

It is evident from these initial experiments that the recovery of compounds of the adrenocortical type from urinary sources is a feasible procedure. While lengthy, the isolation process is relatively simple and the conditions imposed sufficiently mild to preclude largely the possibility that the substances recovered are transformation products. It is to be expected that, with refinement in method, higher yields and a greater number of crystalline compounds may be obtained. The method as here outlined may be applied to the fractionation of urine obtained in pathological states, in pregnancy, and to urine from animal sources.

These data demonstrate that adult males normally excrete small amounts of 17-hydroxy-11-dehydrocorticosterone and lend further support to the thesis that the human adrenal gland elaborates this substance. Qualitatively and quantitatively, this compound could of itself largely account for the biological (glyconeogenic) activity of the cortin fraction. For example, Heard, Sobel, and Venning, using the glycogen deposition method, have reported (8) that normal adult males excrete from 45 to 77 γ of cortin substance per day (expressed in terms of Compound E). In these experiments, the over-all recovery of Compound E may be roughly estimated to be 82 γ per 24 hours, assuming an average 24 hour output of 1500 ml. of urine.

While it is difficult to evaluate isolation experiments in quantitative terms, it seems reasonable to predict that other physiologically active compounds, if found, will be present in comparatively small amounts. Since Compounds II and IV appear to possess two of the structural requisites (the α,β -unsaturated system and the α -ketol side chain) necessary for biological activity, they too may ultimately prove to be active.⁸

⁸ In addition to promoting glycogen deposition, the ability of the cortin fraction to prolong the survival period of the adrenalectomized rat (5, 9) and to increase the tolerance of such animals to cold (10) suggests that it may contain compounds of the 11-desoxy series. If such are present, it may be expected (7) that they would appear in the benzene residues. Neither Compound II nor IV appeared in such a fraction.

Compounds I, III, and V are clearly reduction products. It remains to be determined whether they are elaborated in the gland as such or whether they are derived from other compounds at an extraglandular site. Since they lack the α,β -unsaturated system, it can be predicted that they will have little if any biological activity. The presence of compounds of this type in urine would in part account for the uniformly higher results given by the chemical (colorimetric) methods as contrasted to the biological assay for cortin substances.

It is of interest that these substances are excreted in the free form in some cases (Compound VI (largely) and Compounds I, II, and V) and in a conjugated form in others (Compounds III and IV). The point is of considerable practical importance because it again raises the question of how hydrolysis of the conjugated forms may be most readily and safely achieved. Although acid is employed as the hydrolytic agent in these experiments, it is recognized that, while possibly effective, the procedure may well prove to be destructive to some compounds. The observation frequently made that strong acids, particularly at elevated temperatures, reduce or destroy the activity of cortin fractions suggests that 17-hydroxy-11-dehydrocorticosterone, for example, is acid-labile as well.

While no accurate comparison can be made between these data and those obtained by measuring the chemical or biological properties of extracts prepared before and after hydrolysis at pH 1, it may be noted that the results reported here do not support the statements (8, 11, 12) that hydrolysis increases the yield of extractable active material 2 to 4 times. As may be calculated from Table II, 77 per cent of the crystalline material and 86 per cent of the Compound E isolated were obtained prior to the hydrolysis procedure. The difficulties generally encountered in the treatment of acid-treated urine do not center about the amount or nature of the contaminating substances, for, although the crude extract is dark, the Girard separation together with the benzene-water partitioning effectively removes these.

The author wishes to express his thanks to Dr. Wm. Pearlman of the Department of Biochemistry of this institution for his generous assistance and valued advice, and Dr. T. F. Gallagher and Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, for many helpful suggestions and for determining the infra-red spectra of Compounds I, II, and III.

The data of Heard, Sobel, and Venning (8) include a table illustrating these differences.

SUMMARY

- 1. Normal male urine has been examined for compounds of the adrenocortical type by a procedure that includes extraction with chloroform, recovery of the ketonic fraction, its partitioning between benzene and water, and chromatography of the acetylated residues.
- 2. Of the crystalline compounds recovered, one has been shown to be 17-hydroxy-11-dehydrocorticosterone, which was isolated largely as the free compound in an over-all yield of 55.3 γ per liter.
- 3. Four other crystalline compounds of the adrenocortical type and one other non-crystalline substance have been partially characterized. Three of these are regarded as reduction products and the remaining two as possibly physiologically active compounds.
 - 4. These results have been discussed in relation to other relevant data.

Addendum—Since this paper was submitted, it has been found that the infra-red absorption of Compound I is the same as that given by tetrahydro E acetate $(3(\alpha),21$ -diacetoxy-17(α)-hydroxypregnanetriol-11,20-dione), thus definitely establishing the identity of this compound. The author again wishes to thank Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research for his assistance in this regard.

BIBLIOGRAPHY

- 1. Schneider, J. J., Science, 111, 61 (1950).
- 2. Engstrom, W. W., Yale J. Biol. and Med., 21, 21 (1948).
- 3. Dorfman, R. I., in Pincus, G., and Thimann, K. V., The hormones, New York, 1, 467 (1948).
- Heard, R. D. H., in Pincus, G., and Thimann, K. V., The hormones, New York, 1, 549 (1948).
- Venning, E. H., Hoffman, M. M., and Browne, J. S. L., Endocrinology, 35, 49 (1944).
- 6. Mason, H. L., and Sprague, R. G., J. Biol. Chem., 175, 451 (1948).
- 7. Mason, H. L., Myers, C. S., and Kendall, E. C., J. Biol. Chem., 114, 613 (1936).
- 8. Heard, R. D. H., Sobel, H., and Venning, E. H., J. Biol. Chem., 165, 699 (1946).
- 9. Dorfman, R. I., and Horwitt, B. N., Federation Proc., 2, 60 (1943).
- Dorfman, R. I., Shipley, R. A., Schiller, S., and Horwitt, B. N., Endocrinology, 38, 165 (1946).
- Daughaday, W. H., Jaffe, H., and Williams, R. H., J. Clin. Endocrinol., 8, 166 (1948).
- 12. Venning, E. H., Kazmin, V. E., and Bell, J. C., Endocrinology, 38, 79 (1946).

SYNTHETIC ACTION OF PHOSPHATASE*

II. TRANSPHOSPHORYLATION BY ALKALINE PHOSPHATASE IN THE ABSENCE OF NUCLEOTIDES

BY OTTO MEYERHOF AND HARRY GREEN

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, September 20, 1949)

Axelrod (1) recently reported that a direct transfer of phosphate occurred from some aryl phosphates to certain aliphatic alcohols in the presence of citrus phosphatase and some other acid phosphatases. The phosphate did not pass through the inorganic stage. This was indicated by the fact that inorganic phosphate did not esterify the acceptor compound under the conditions in which it was phosphorylated by nitrophenyl phosphate. The direct transfer was, moreover, confirmed by experiments with P³² (2). This phenomenon was observed not to occur with the biological sugars or in the presence of ordinary alkaline phosphatase.

We have found, however, that a similar transfer occurs readily with alkaline phosphatase and with the common biological polyalcohols (glucose, fructose, and glycerol) in the presence of phosphocreatine, phosphopyruvate, and glucose-1-phosphate. In these cases it can be observed only with P32, because, in contrast to the system studied by Axelrod, the ester phosphates formed remain in enzymatic equilibrium with the inorganic phosphate and with the free polyalcohol from which the ester is formed. There is no excess formation of ester over that allowed by the thermodynamic equilibrium. The phosphate transfer proceeds only from a higher level of phosphate energy to a lower level. It is most pronounced in the system phosphopyruvate or phosphocreatine to glycerol. These transfers attain 100 per cent; that is, all synthesis of glycerophosphate over that of the control in the absence of the phosphate donor occurs by transfer. This was already surmised by us (3) when we observed an increase of several hundred per cent in the speed of synthesis of glycerophosphate in the presence of phosphocreatine. Glucose-1-phosphate can also be used as P donor: fructose and glucose instead of glycerol can be used as acceptors. We have, finally, found such a phosphate transfer from phosphocreatine

^{*} This work was aided by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation. A preliminary note has appeared (Science, 110, 503 (1949)).

to glycerol in an enzymatic mixture with glycerophosphate at equilibrium, if labeled phosphocreatine is added. Even if the system is not at equilibrium, by virtue of a several fold excess of glycerophosphate so that the latter breaks down to attain the equilibrium distribution, the transfer of P from phosphocreatine to glycerophosphate can still be demonstrated.

Methods

All incubations were conducted at 38°.

Phosphate was determined by the method of Fiske and Subbarow (4) as modified by Lohmann and Jendrassik (5).

Inorganic phosphate was precipitated by magnesia mixture and removed as MgNH₄PO₄.

The amount of synthesized organic phosphate, or, as in the equilibrium and hydrolysis studies of glycerophosphate, the amount of glycerophosphate remaining, was determined as the difference between the total organic phosphate and that of the added phosphate donor remaining. In every experiment a control incubation was run simultaneously in order to follow the rate of hydrolysis of the added phosphate donor in the absence of the material being phosphorylated. The added phosphate donor was determined by acid hydrolysis (phosphocreatine and glucose-1-phosphate) or by alkaline hypoiodite oxidation (phosphopyruvate).

Materials-

Phosphate acceptors. Merck's anhydrous glucose, Merck's crystalline levulose, Baker's analyzed glycerol.

Phosphate donors. Phosphocreatine (barium salt) labeled with P³² was prepared enzymatically according to the method of Meyerhof, Schulz, and Schuster (6), and recrystallized to constant purity, 96 to 97 per cent of labile phosphorus. Glucose-1-phosphate (dipotassium salt) labeled with P³² was prepared enzymatically according to the method of Kiessling (7). It was best crystallized by the slow addition of alcohol to the hot aqueous solution until persistent turbidity and by permitting the mixture to come slowly to room temperature. Tiny white crystals were obtained containing 99 to 100 per cent of 10 minute-hydrolyzable phosphorus. Phosphopyruvate (barium salt) was a synthetic preparation made by Kiessling (8) about 10 years ago and purified in our laboratory (9). The purity was 99 per cent by the alkaline hypoiodite method of Lohmann and Meyerhof (10).

Na-glycerophosphate (Heyden Chemical Corporation) 60 per cent α , 40 per cent β ester.

 $Ca-\alpha$ -glycerophosphate (Bios Laboratories, Inc.) 97 per cent α ester.

Radioactive inorganic phosphate. When used in the experiments with phosphopyruvate, the sample was hydrolyzed in 2 N HCl for 20 minutes and treated with a few drops of dilute KMnO₄ for 5 minutes at the reflux

temperature. This procedure removed the metaphosphate and phosphite, with which the orthophosphate (P³²) from the Isotopes Division, Oak Ridge, is often contaminated. After the treatment only a negligible amount of P³² not precipitated by magnesia mixture remained.

Alkaline intestinal phosphatase was purified according to Schmidt and Thannhauser (11).

Handling of Phosphates for Counting

All phosphate species were recovered in the form of MgNH₄PO₄. The stable organic phosphates (glucose-6-phosphate, fructose phosphate, and glycerophosphate) were decomposed by incineration or by phosphatase hydrolysis, while the labile phosphate of the added phosphate donor was split by suitable means as mentioned above. Carrier inorganic phosphate was added prior to precipitation with magnesia mixture in order to dilute the activity when necessary. The last trace of residual activity was similarly removed by the addition of carrier inorganic phosphate and subsequent removal by magnesia mixture. The precipitates of MgNH₄PO₄ were washed three times with 3 per cent NH₄OH and twice with acetone and plated onto a tin plate from an acetone suspension.

After having been counted, the plated samples were washed off the plates with H_2O , dissolved in dilute acid, and analyzed for inorganic phosphate. From the ratio of labeled phosphate species to added carrier phosphate and from the total activity of this mixture, the specific activity of the undiluted labeled phosphate species was readily calculated: A = micrograms of P of the labeled phosphate species; B = micrograms of P of added carrier phosphate; C = micrograms of P of the counted sample; N = the activity of C. Therefore, the specific activity of labeled phosphate species C = micrograms of P of the counted sample; C = micrograms of P of the counted sample sample

EXPERIMENTAL

All incubations were carried out at 38° and pH 8.5 in a volume varying from 0.5 to 1.1 ml. All calculations in the tables are made on the basis of 1 ml.

A typical experiment on the transphosphorylation of glycerol by means of radioactive phosphocreatine is described in detail as illustrative of the general procedure. In the other experiments the conditions and procedure were essentially the same; therefore, only the modifications of this general procedure will be described.

Glycerol, Radioactive Phosphocreatine (Table I, Experiment I)—The fol-

¹ The quantitative recovery of the MgNH₄PO₄ from the plate in this procedure is indicated by the fact that the residual activity of the washed plate could be accounted for by the background count.

lowing solutions were incubated at 38° at pH 8.5 for 15 minutes: (a) 0.1040 gm. of glycerol, 0.12 ml. of Na₂HPO₄ (2.75 M), 0.50 ml. of H₂O, and 0.05 ml. of purified intestinal phosphatase (activity, 3280 units per ml. as defined by Schmidt and Thannhauser); (b) same as (a) but containing 535 γ of P of labile radioactive phosphocreatine and 21 γ of P of non-

		T	ABLE I	
A.	Transphosphorylation	of	Glycerol by P32-Phospho	creatine

Experiment	,	Initial	molar concen	Glycero-	Phosphocrea-		
No.	ac	Glycerol	Inorganic P	Phospho- creatine	phosphate formed	tine split	
					μ Μ	per cent	
Ia		1.52	0.438	0	0.54	ĺ	
Ib		1.52	0.438	0.023	1.70	18.7	
Ic		0	0.438	0.023		21.5	
IIa		1.63	0.447	0	1.43		
\mathbf{IIb}		1.63	0.447	0.025	4.13	25.3	
Hc		0	0.447	0.025		30.5	

B. Specific Activity of (b) Samples Counts per minute per microgram of P.

		Phosphate species									
Experiment No.	Acceleration of synthesis	Phosphocreatine			Inorg	anic P	Glycerophosphate				
		0 min.	15 min.	30 min.	15 min.	30 min.	15 min.	30 min.			
	per cent										
Ib	218	2412	2210		15		(1738)				
				1			1487*				
Пр	188	1090		895		9		(707)			
								614*			

^{*} Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

labile radioactive organic phosphate;² (c) same as (b) but with the glycerol replaced by an equal volume of H_2O .

The solutions were deproteinized with 1.0 ml. of ice-cold 5 per cent trichloroacetic acid, chilled, and immediately made ammoniacal with 1.0 ml. of 12 n NH₄OH. The inorganic phosphate was precipitated by magnesia mixture in a total volume of 15.0 ml. and removed by filtering through a sintered glass funnel. The filtrates were analyzed for inorganic and organic phosphate. The determination of direct phosphate under the

² The non-labile organic impurity varied, in different samples, from 3 to 4 per cent of the total organic P.

conditions of Fiske and Subbarow as modified by Lohmann and Jendrassik gave the amount of phosphocreatine unsplit in incubations (b) and (c). In the latter sample, moreover, the difference between the total organic and phosphocreatine phosphate represented the amount of non-labile impurity of phosphocreatine remaining as stable organic phosphate. remaining filtrate (13.0 ml.) of incubation sample (b), containing the radioactive glycerophosphate, was boiled to rid it of excess NH₃, acidified with 2.0 ml. of 12 N HCl, and incubated at 38° for 30 minutes in order to hydrolyze the unsplit phosphocreatine. Carrier inorganic phosphate (60 mg.) was added and the total inorganic phosphate precipitated by magnesia mixture and removed by filtration through a sintered glass funnel. precipitate is designated as the phosphocreatine phosphate species, while the first precipitate of the inorganic phosphate is the inorganic phosphate The filtrate of the above was again treated with carrier inorganic phosphate (30 mg.) and the treatment with magnesia mixture repeated. The latter precipitate was discarded. The filtrate containing the glycerophosphate and non-labile phosphate from the phosphocreatine was boiled to free it from excess NH3 and analyzed for inorganic and organic phosphate. To the remaining volume (18 ml.) were added 10 ml. of borate buffer (pH 9.1); the mixture was adjusted to pH 9.3 and incubated overnight at 38° with 1640 Schmidt units of phosphatase. The increase in the inorganic phosphate (95 to 100 per cent of the organic phosphate initially present) corresponded to the glycerophosphate species, and was derived from both the glycerophosphate and the non-labile organic phosphate originally present in the phosphocreatine. To the remaining solution containing 17 γ of P from the glycerophosphate and 9 γ of P from the non-labile impurity of phosphocreatine was added carrier inorganic phosphate (5.0 mg.), and the total inorganic phosphate was precipitated with magnesia mixture and removed as MgNH₄PO₄ (glycerophosphate species). As a standard for comparison of activities a sample of the stock radioactive phosphocreatine solution used in the incubations was hydrolyzed in 1 N HCl at 38° for 30 minutes, carrier inorganic phosphate added, and the total inorganic phosphate precipitated by magnesia mixture and removed as MgNH₄PO₄.

All precipitates of MgNH₄PO₄ prior to counting were washed three times with 3 per cent NH₄OH and twice with acetone. The results are given in Table I, Experiment I. The total activity accounted for was 93 per cent (see Table II).

Fructose, Radioactive Phosphocreatine (Table III)—Removal of excess NH₃ by boiling and the hydrolysis of the fructose phosphates by phosphatase at 38° were performed in an atmosphere of nitrogen gas in order to avoid discoloration due to oxidation by air.

TABLE II
Calculation of Activity Balance

P species	P	Specific activity	Total activity	
	γ			
Input			İ	
Phosphocreatine (+ impurity)	546	2412	1,315,000	
Recovered				
Inorganic	10,270	14.8	152,000	
Labile phosphocreatine unsplit	435	2210	962,000	
Glycerophosphate fraction (+ phosphocreatine impurity)	61	1738	106,000	
Sum		93	1 ,220 ,000	

Experiment	Enzyme units	Initial	molar concer	itration	Fructose phos-	Phosphocreatine split	
No.	per ml.	Fructose	Inorganic P	Phospho- creatine	phate formed		
					μ Μ	per cent	
Ia	372	2.06	0.443	0	0.55		
${f Ib}$	372	2.06	0.443	0.028	1.87	12.4	
${f Ic}$	372	0	0.443	0.028		30.0	
IIa	372	2.06	0.443	0	0.62		
ПЬ	372	2.06	0.443	0.028	1.72	13.3	
IIc	372	0	0.443	0.028		31.5	

B. Specific Activities of (b) Samples

Counts per minute per microgram of P.

		Phosphate species						
Experiment No.	Acceleration of synthesis	Phospho	ocreatine	Inorganic P	Fructose phos- phate			
		0 min.	15 min.	15 min.	15 min.			
	per ceni							
Ib	227	1828	1750	13	(1190) 556*			
IIb.	175	2380	2145	15	(1108) 607*			

^{*}Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

Glycerol, Radioactive Glucose-1-phosphate (Table IV)—The glucose-1-phosphate unsplit at the end of the incubation was decomposed by hydrolysis in 1 n HCl at 100° for 15 minutes. The inorganic phosphate liberated was precipitated by magnesia mixture and worked up as described.

Glycerol or Glucose, Phosphopyruvate, Radioactive Inorganic Phosphate (Table V)—In this series of experiments the transphosphorylation is demonstrated not by an excess of activity in the synthesized ester but by a lower

Table IV

A. Transphosphorylation of Glycerol by P^{s_2} -Glucose-1-phosphate; Incubations for 15 Minutes

Experiment	Enzyme units	Initial	molar concer	itration	Glycerophos-	Glucose-1-phos-
No.	per ml.	Glycerol	Inorganic P	Glucose-l- phosphate	phate formed	phate split
					μ μ	per ceni
Ia	138	1.97	0.524	0	0.57	
Ib	138	1.97	0.524	0.014	1.15	42.5
Ic	138	0	0.524	0.014		53.2
IIa	93	1.92	0.509	0	0.62	
\mathbf{IIb}	93	1.92	0.509	0.027	1.52	8.9
He	93	0	0.509	0.027		20.5

B. Specific Activities of (b) Samples Counts per minute per microgram of P.

			ate species			
Experiment No.	Acceleration of synthesis	Glucose-1	-phosphate	Inorganic P	Glycerophos- phate formed	
		0 min.	15 min.	15 min.	15 min.	
	per ceni					
${f I}{f b}$	100	1935	1890	18	543	
IIb	145	2400	2070	15	673	

activity compared with that of the inorganic phosphate species. The highly active inorganic phosphate fraction after removal from the incubation mixture by magnesia mixture was dissolved in dilute HCl, analyzed for phosphate, treated with a known amount of carrier inorganic phosphate, and reprecipitated as MgNH₄PO₄. The phosphopyruvate fraction, on the other hand, was completely free of any activity, and was, therefore, discarded after removal. The phosphopyruvate was decomposed by alkaline hypoiodite; in the presence of glucose, however, it was necessary to add an excess of hypoiodite to oxidize completely both the glucose and the phosphopyruvate.

Glycerophosphate, Radioactive Phosphocreatine (Tables VI and VII)—A several fold excess of glycerophosphate over the equilibrium value was used in the hydrolysis experiment (Table VII) in order to produce a

Table V

A. Transphosphorylation of Glycerol and Glucose by Phosphopyruvate with P**Inorganic Phosphate

Experiment	Enzyme	Time of		Initial n	olar conc	entration	Acceptor	Phospho-
No.	units per ml.	incubation	P acceptor	P acceptor	Inor- ganic P	Phospho- pyruvate	phosphate formed	pyruvate split
		min.	Address of the second of the				μ Μ	per ceni
la	213	15	Glycerol	1.47	0.432	0	0.58	
Ib	213	15	"	1.47	0.432	0.021	4.52	65
\mathbf{Ic}	213	15		0	0.432	0.021		77
IIa	213	15	Glycerol	1.47	0.432	0	0.63	
IIb	213	15	"	1.47	0.432	0.020	4.23	67
IIc	213	15		0	0.432	0.020		77
IId	213	150	Glycerol	1.47	0.432	0	3.27	
IIe	213	150	"	1.47	0.432	0.020	10.1	100
IIIa	365	15	Glucose	2.06	0.423	0	0.74	
IIIb	365	15	"	2.06	0.423	0.024	5.38	67
IIIc	365	15		0	0.423	0.024		82
IIId	365	150	Glucose	2.06	0.423	0	4.88	
IIIe	365	150	"	2.06	0.423	0.024	8.95	100

B. Specific Activities of (b) and (e) Samples Counts per minute per microgram of P.

		Phosphate species								
Experiment No.	Acceleration of synthesis	Inorganic P			Glycerop	hosphate	Glucose-6-phosphate			
		0 min.	15 min.	150 min.	15 min.	150 min.	15 min.	150 min		
	per ceni									
Ιb	680	1174	1123		242		}			
\mathbf{IIb}	570	587	548		120					
IIe		587		437		310				
IIIb	630	527	490				84			
IIIe		527		400				334		

substantial hydrolysis and, at the same time, permit an accurate analysis of the glycerophosphate remaining at the end of the experiment. In addition to the control which allowed us to follow the rate of splitting of phosphocreatine in the absence of glycerol and glycerophosphate, another control containing both glycerophosphate and phosphocreatine but not glycerol was run simultaneously. The latter was included for the purpose

of determining whether any direct isotopic exchange had taken place between the radioactive phosphocreatine and the glycerophosphate in the absence of free glycerol. In both experiments (equilibrium and hydrolysis) because of the relatively large amounts of glycerophosphate present at the end of the incubation, it was necessary to wash the first precipitate of MgNH₄PO₄ with dilute NH₄OH to avoid a considerable loss of glycero-

Table VI

A. Phosphate Transfer at Enzymatic Equilibrium of Glycerophosphate in Presence of Pre-Phosphocreatine

T	Enzyme units per ml.	Time of incubation	Init	ial molar	Glycero-	Phospho-		
Experiment No.			Glycerol	In- organic P	Phospho- creatine	Glycero- phos- phate*		creatine split
		min.					м	per cent
Ia	213	15	1.86	0.346	0	0.025	0.024	
\mathbf{Ib}	213	15	1.86	0.346	0.022	0.025	0.024	8.0
Ic	213	15	0	0.346	0.022	0		10.5
Ha	213	150	1.86	0.346	0	0.025	0.024	
\mathbf{H}	213	150	1.86	0.346	0.022	0.025	0.023	50.0
Hc	213	150	0	0.346	0.022	0		68.0

	Phosphate species										
Experiment No.	Phosphocreatine			Inorg	anic P	Glycerophosphate					
	0 min.	15 min.	150 min.	15 min.	150 min.	15 min.	150 min.				
Ib	1240	1210		7.6		(151)					
					l	79†					
\mathbf{IIb}	1240		1218		30.5		(233)				
			1				126†				

B. Specific Activities of (b) Samples

phosphate by occlusion. Similarly, it was found more practicable to decompose the glycerophosphate fraction by incineration rather than by phosphatase hydrolysis.

Results

When we recently observed (3) a high percentage increase of the rate of synthesis of glycerophosphate by the addition of phosphocreatine (250 per cent increase), of glucose-1-phosphate (130 per cent), and of fructose-

^{*} Calcium salt of α -glycerophosphate (Bios), 97 per cent α .

[†] Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

1-phosphate (100 per cent), it was surmised that this increase might result from a transphosphorylation from phosphate donors of higher phosphate energy to acceptors of lower energy. It could easily be demonstrated by use of P³² that this was actually the case. When the phosphate donor was

Table VII

A. Phosphate Transfer during Enzymatic Hydrolysis of Glycerophosphate in Presence of Pss-Phosphocreatine

Experiment No.	Enzyme units per ml.	Initial molar concentration				Glycerophos-	Phosphocrea-
		Glycerol	Inorganic P	Phospho- creatine	Glycero- phosphate*	phate split	tine split
						per coni	per cons
Ia	219	1.53	0.007	0	1.40	70.5	
Ib	219	1.53	0.007	0.022	1.40	35. 4	74.0
Ic	219	0	0.007	0.022	0		94.0
IIa	219	1.53	0.024	0	1.40	48.5	
IIb	219	1.53	0.024	0.022	1.40	18.5	56.0
IIc	219	0	0.024	0.022	0		75.0
IId	219	0	0.024	0.022	1.40	70.5	65.0

B. Specific Activities of (b) and (d) Samples Counts per minute per microgram of P.

	Phosphate species					
Experiment No.	Phosph	ocreatine	Inorganic P	Glycerophosphate		
	0 min.	15 min.	15 min.	15 min.		
Ib	1195	1200	449	(557) 527†		
IIb	1250	1293	280	(443) 378†		
IId	1250	1260	380	(93) 0.71†		

^{*} Sodium salt of glycerophosphate (Heyden), 60 per cent α ; 40 per cent β .

labeled, the synthesized glycerophosphate or fructose phosphate became highly radioactive, while the specific activity of the inorganic phosphate resulting from a partial split of the phosphate donor remained very low. In the other case, when the inorganic phosphate was labeled with P²², the synthesized glycerophosphate or glucose phosphate in the presence of unlabeled phosphopyruvate had a very low radioactivity for the first 15 minutes, proving that most of the esterified phosphate did not come from the inorganic phosphate but from the phosphate donor.

[†] Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

In order to calculate the relative effectiveness of the transphosphorylation compared with the synthesis of the ester from inorganic phosphate, the acceleration of synthesis in the presence of the phosphate donor must be taken into account. This acceleration, as can be seen from the B sections of the tables, is quite different for the various combinations: In the early period of the incubations (15 minutes) it is 600 per cent from phosphopyruvate to glycerol or glucose, 200 per cent from phosphocreatine to glycerol or fructose, and 100 per cent from glucose-1-phosphate to glycerol. This increase in speed has to be compared with the specific activity of the synthesized ester in relation to the activity of the inorganic phosphate and the phosphate donor. This is very simple when the phosphate donor is unlabeled and the inorganic phosphate labeled. In the experiments with phosphopyruvate (Table V) the 15 minute values of glycerophosphate and of glucose phosphate have respective activities of 20 and 17 per cent of the activity of the inorganic phosphate. Therefore, 80 and 83 per cent, respectively, of the synthesized esters was formed by transphosphorylation. For a 600 per cent increase in speed the theoretical figure is 85.5 per cent. However, the kinetic exchange between the newly formed ester and the inorganic phosphate must tend to decrease this value the longer the incubation lasts. We, therefore, conclude from these experiments that, in this case, the acceleration stems wholly from transphosphorylation.

When the phosphate donor contained the labeled phosphate, a correction was necessary for the non-labile impurities. These were negligible in the glucose-1-phosphate, but amounted to 3 to 4 per cent in the phosphocreatine. These impurities had the same specific activity as the phosphocreatine and bore more weight the smaller the amount of synthesized ester. The values measured directly were corrected accordingly (values indicated in the tables). For example, in Experiment I of Table I, the corrected value for glycerophosphate gave 1487 counts² compared with 2210 counts for phosphocreatine and 15 for inorganic phosphate (15 minutes). The acceleration of the synthesis was 218 per cent. Therefore, we expect 31.5 per cent synthesized from inorganic phosphate and 68.5 per cent from phosphocreatine. Indeed, 1487 is 67 per cent of 2210. Here, again, excess synthesis over the control is wholly explained by transphosphorylation.

³ This value is calculated from the measured non-labile impurity remaining at the end of incubation and from the specific activity of the phosphocreatine species for the same time period. This presupposes that the activity of the non-labile impurity exchanges in the same way as that of the phosphocreatine itself. Because the possible exchange of the P²² in the impurity could not be established, this calculation under the circumstances seemed to be based upon the simplest assumption.

If we apply the same reasoning to the transphosphorylation from phosphocreatine to fructose and also to that from glucose-1-phosphate to glycerol, we find the transphosphorylation to be less complete. The acceleration in Experiment II of Table III was 175 per cent; 64 per cent should, therefore, be formed by transphosphorylation. But the value was 607/2145 or 28 per cent; similar results were obtained in the other experiments. The acceleration with glucose-1-phosphate was 100 per cent (Table IV). Instead of an expected activity of the synthesized ester of 50 per cent that of glucose-1-phosphate we found only 29 per cent. It seems, therefore, that in the cases in which the acceleration is smaller, it is only in part, but not wholly, explained by transphosphorylation.

As the results of Tables VI and VII indicate, the exchange of P³² from phosphocreatine to glycerophosphate can be demonstrated in a state of near equilibrium and, to a smaller extent, even during dephosphorylation of glycerophosphate. In the former case, the activity of the remaining glycerophosphate increases with time, but less than would be expected if the rate of exchange were uniform. With dephosphorylation of glycerophosphate the excess activity over that of the inorganic phosphate is small because the latter increases rapidly with time as a result of the splitting of the active phosphocreatine. It can, however, still be seen with as low a concentration of inorganic phosphate as 0.007 m (Table VII, Experiment I). In the absence of free glycerol the glycerophosphate was split, practically without any reverse reaction, and did not take up any measurable activity, not even from the inorganic phosphate (Table VII, Experiment IId).

DISCUSSION

According to the experiments described in the foregoing pages the transfer of phosphate by means of phosphatase seems to be a common phenomenon. Although we have not investigated types of phosphatases other than the alkaline intestinal phosphatase, we would expect this phenomenon to be general. The fact that in the balance the transfer goes always from higher energy phosphate to lower energy phosphate is to be expected from thermodynamic considerations, if all components are in free exchange with one another. Kinetically, the opposite transfer also occurs, but it must be relatively slow and it is observed in our experiments only by a small dilution (5 to 10 per cent) of the P³² in the phosphate donor during the time of incubation. This percentage dilution, however, is not far from the over-all experimental error.

The findings of Axelrod (1) that some, but not all, acid phosphatases and no alkaline phosphatase allow the esterification of certain primary aliphatic alcohols in the presence of aryl phosphates, like nitrophenyl

and phenyl phosphate, must be regarded as a special and exceptional case in the light of our results. If such an accumulation of an aliphatic phosphate ester occurs in spite of the phosphatase, which would normally hinder this tendency, it shows that the phosphatases in question are not able to split readily the specific esters which have formed. For example, the equilibrium constant of methyl phosphate, calculated from the measurements of McVicar (12), is $K = (\text{methanol} \times \text{phosphate})/(\text{water} \times \text{phosphate})$ methyl phosphate) = about 1.0 at pH 8.2 and probably greater than 2.0 at pH 6. This means that with 3.5 m methanol, 0.018 m inorganic phosphate, and 47.5 m H₂O only 8 \times 10⁻⁴ m methyl phosphate can form in the equilibrium at pH 6. But in the experiments of Axelrod ((2) p. 297) 1.2×10^{-2} m methyl phosphate, 15 times as much, was formed. This apparently is "trapped" because the enzyme cannot readily split it.4 In the case of the alkaline phosphatase such accumulation is impossible and the transfer, although it occurs as well as with the citrus fruit phosphatase, is with non-isotopic components visible only as an increase in speed of synthesis before the final equilibrium distribution is obtained. invisible transfer can be studied directly by the use of P32 (see (13)).

The shift of the phosphate from the higher energy level to the lower is the more rapid the larger the energy difference. From phosphopyruvate to glycerol the increase in speed is 7-fold, from phosphocreatine 3-fold, and from glucose-1-phosphate 2-fold. The respective ΔF^0 values of the phosphate bond are as follows: for phosphopyruvate, -16,000 calories; for phosphocreatine, -11,500 calories; for glucose-1-phosphate, -4800 calories; for glycerophosphate, -2200 calories. These values together with those of the P^{32} -phosphate exchange presented here show that the energy differences between the phosphate donors and acceptors determine the extent of transfer per unit of time.

Because the concentrations of phosphate and polyalcohol must be high for an actual synthesis of the phosphate esters to occur, it is uncertain whether this mechanism of transfer has a biological application. However, as the experiments of Table VII show, the transfer can be observed even against the direction of the final equilibrium and with as low a phosphate concentration as 0.007 m. In any case, it is important from a theoretical view-point. It shows that the phosphatese reacts with phosphate acceptors as well as with phosphate donors: if no acceptor is present, the phosphate becomes directly liberated as inorganic phosphate; if an acceptor is present the phosphate group is at first transferred from the higher energy to the lower energy level before being split off.

4 It was observed by Axelrod (cf. (1) p. 3) that methyl phosphate was split by citrus phosphatase 7 times more slowly than was nitrophenyl phosphate at an equivalent concentration.

We thank Mr. John W. Harris, Jr., for assistance in the counting of the radioactive samples.

SUMMARY

The increase in the speed of synthesis of biological phosphate esters in the presence of alkaline intestinal phosphatase, if phosphate compounds of higher phosphate energy are added, is caused by transphosphorylation. This is proved by the use of phosphate donors, labeled with P³² and unlabeled inorganic phosphate, or by using unlabeled donors and radioactive inorganic phosphate.

In the system P³²-phosphocreatine and glycerol, 67 per cent of the total synthesis is caused by transphosphorylation, while from the increase in speed 68.5 per cent would be expected. In the system phosphopyruvate and glycerol or glucose and labeled inorganic phosphate, 80 and 83 per cent, respectively, of the synthesis is caused by transphosphorylation, while from the speed 85.5 per cent is expected.

In the systems phosphocreatine and fructose and P³²-glucose-1-phosphate and glycerol only half of the increase in speed can be explained by transphosphorylation, according to the specific activities of the reactants and products.

This transphosphorylation can be demonstrated also when glycerophosphate, glycerol, and phosphate are in equilibrium in the presence of labeled phosphocreatine and even if glycerophosphate, in the presence of glycerol, breaks down, owing to phosphate concentrations below the equilibrium value.

BIBLIOGRAPHY

- 1. Axelrod, B., J. Biol. Chem., 172, 1 (1948).
- 2. Axelrod, B., J. Biol. Chem., 176, 295 (1948).
- 3. Meyerhof, O., and Green, H., J. Biol. Chem., 178, 655 (1949).
- 4. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., 68, 375 (1925).
- 5. Lohmann, K., and Jendrassik, L., Biochem. Z., 178, 419 (1926).
- 6. Meyerhof, O., Schulz, W., and Schuster, P., Biochem. Z., 293, 309 (1937).
- 7. Kiessling, W., Biochem. Z., 298, 421 (1938).
- 8. Kiessling, W., Ber. chem. Ges., 68, 597 (1935); 69, 2331 (1936).
- 9. Meyerhof, O., and Oesper, P., J. Biol. Chem., 179, 1371 (1949).
- 10. Lohmann, K., and Meyerhof, O., Biochem. Z., 273, 60 (1934).
- 11. Schmidt, G., and Thannhauser, S. J., J. Biol. Chem., 149, 369 (1943).
- 12. McVicar, G. A., Thesis, University of Toronto (1934).
- Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, H., Biochem. Z., 298, 396 (1938).

STUDIES ON ADENOSINETRIPHOSPHATASE OF MUSCLE*

III. THE LIPOPROTEIN NATURE OF THE MAGNESIUM-ACTIVATED ADENOSINETRIPHOSPHATASE

BY W. WAYNE KIELLEY AND OTTO MEYERHOF

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, September 20, 1949)

In the initial work (1) on the partial isolation and properties of a new magnesium-activated ATPase¹ of muscle, it was observed that the enzyme preparations contained a large amount of organic phosphorus, most of which was lipide phosphorus. The relatively small amount of nucleic acid phosphorus could be removed with ribonuclease without impairing the activity of the ATPase. Attempts to separate the enzyme from the lipide material were unsuccessful. The present report is the result of continued attempts to purify the ATPase by separation of the protein and lipide components. These experiments have produced substantial evidence that the association between lipide and protein is essential for the characteristic ATPase activity.

Methods

Preparation of Mg-ATPase—The procedure used in this work was a modification of that reported previously (1). The composition of the extracting solution was as follows: 0.1 m KCl, 0.04 m NaHCO₃, 0.01 m Na₂CO₃, and 0.001 m KCN. The muscle was first run through a meat chopper, then mixed with 5 volumes of the extracting solution, and agitated in a Waring blendor for 1 minute. After standing about 30 minutes, the suspension was centrifuged at 4500 r.p.m. for 15 minutes. The residue was suspended in 5 volumes of the extracting solution and centrifuged as before. The procedure was then repeated a second time. The three extracts were combined, diluted with an equal volume of 0.001 m KCN, centrifuged to remove myosin, and the ATPase precipitated with

- * This work was aided by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation.
- † Postdoctorate Research Fellow, National Institutes of Health, Bethesda, Maryland. Present address, Department of Physiology, Tufts College Medical School, Boston, Massachusetts.
- ¹ ATP = adenosine triphosphate; ATPase = adenosinetriphosphatase; Mg-ATPase = magnesium-activated adenosinetriphosphatase.

 $(\mathrm{NH_4})_2\mathrm{SO_4}$ at 35 per cent saturation. After centrifuging, the precipitate was washed once with 35 per cent saturated $(\mathrm{NH_4})_2\mathrm{SO_4}$ in order to pack it more firmly and then redissolved in a 1:1 dilution of the extracting solution. The $(\mathrm{NH_4})_2\mathrm{SO_4}$ precipitation was repeated once. The precipitate was dissolved in a mixture of 0.06 m histidine, 0.2 m KCl, and 0.001 m KCN, the volume being about equal to the original weight of muscle. The manipulations up to this point were carried out as a continuous operation. After clarifying the final solution of the ATPase by centrifuging at $6000 \times g$ for 20 minutes, the ATPase was sedimented by centrifuging at $18,000 \times g$ for 2 to 3 hours. The precipitate was dissolved in about one-fifth the original volume and in the same histidine-KCl-KCN buffer.

Since large amounts of the enzyme were required for these experiments, rabbit muscle was used. The preparations from this source have never been as satisfactory with regard to both yield and purity as those obtained from rat muscle. $Q_{\rm P}$ values² for the preparations used in the work reported here varied from 5000 to 8000. The procedure given above for the preparation of the Mg-ATPase is somewhat more convenient for the quantities of muscle used (about 300 gm.), but the yield is only 80 per cent of that obtained by the former method.

Preparation of Clostridium welchii Lecithinase—Toxic filtrates of C. welchii type A cultures were furnished by the Lederle Laboratories.³ The solutions were first dialyzed against distilled water. The enzyme was then adsorbed on calcium phosphate at pH 6.5 in the presence of 25 per cent acetone, centrifuged, and eluted with 15 to 20 per cent saturated $(NH_4)_2SO_4$, according to the procedure of van Heyningen (2). This procedure was repeated once, thereby bringing an initial volume of 1 liter down to about 15 ml. After dialysis the preparation was dried from the frozen state. A well dialyzed preparation had an activity of about 15 to 20 units per mg., a unit being defined as the amount of enzyme producing 1 μ M of phosphorylcholine in 10 minutes at pH 7.3 to 7.4 and 38° in the presence of about 200 γ of lecithin P in a total volume of 1.3 ml.

It has been demonstrated by Macfarlane and Knight (3) that this lecithinase acts upon lecithin to produce phosphorylcholine and a diglyceride. It has been further demonstrated by Zamecnik, Brewster, and Lipmann (4) and Macfarlane (5) that the lecithinase will not act on lysolecithin, glycerophosphorylcholine, or "cephalin," but attacks only lecithin and sphingomyelin. Following the suggestion of Zamecnik et al. (4), we dissolved our lecithinase preparations in 1 per cent serum albumin (crystalline

² Q_P = microliters of H₂PO₄ split off per mg. of protein per hour at 38°.

³ We thank Dr. I. S. Danielson of the Lederle Laboratories Division, American Cyanamid Company, for supplying this material.

bovine serum albumin obtained from Armour and Company) in order to stabilize the enzyme in solution.

Determination of ATPase Activity—The procedure was identical with that used previously (1). The system consisted of 0.1 ml. of 0.15 m MgCl₂, 1.0 ml. of histidine buffer (0.1 m, pH 6.8 to 6.9 at 38°), 0.5 ml. of ATP solution (approximately 660 γ of 7 minute-hydrolyzable P per ml.), 0.2 ml. of the ATPase solution, and H₂O to a total volume of 3.0 ml. The time was 5 minutes and the temperature 38°. The reaction was stopped by addition of 3.0 ml. of 5 per cent trichloroacetic acid. After centrifuging, 2.0 ml. aliquots were analyzed for inorganic phosphorus.

Determination of Lecithinase Activity—Lecithin was prepared from eggs by the cadmium salt procedure of Levene and Rolf (6). With these preparations it was observed that satisfactory emulsions could only be obtained by sonic vibration (9000 cycles). With the Raytheon vibrator4 20 minutes treatment at room temperature was sufficient to produce a slightly opalescent emulsion of 2 to 3 per cent lecithin. The system employed for determination of activity consisted of 0.3 ml. of 0.1 m histidine (pH 7.3 to 7.4 at 38°), 0.1 ml. of 0.05 or 0.075 M CaCl₂, 0.3 ml. of 2 per cent lecithin (approximately 200 γ of P), 0.2 ml. of the lecithinase in 1 per cent serum albumin, and water to a total volume of 1.3 ml. The time of incubation was 10 minutes and the temperature 38°. Two different procedures were used in stopping the reaction and in making the analysis for the product. The first of these involved the use of trichloroacetic acid, centrifuging, filtering, and determining phosphorus on an incinerated aliquot of the filtrate. The second consisted of heat denaturation, centrifugation, filtration, digestion of an aliquot of the filtrate with alkaline phosphatase to hydrolyze the phosphorylcholine, and determining inorganic P after the phosphatase treatment. The preparation of alkaline phosphatase from intestinal mucosa followed the procedure of Schmidt and Thannhauser (7).

In the first method, 0.2 ml. of 40 per cent trichloroacetic acid was added to the incubation mixture. After centrifuging and filtering, 0.5 ml. of the filtrate was incinerated and the inorganic phosphorus determined. In the second procedure, the incubation tubes were placed in a boiling water bath for 5 minutes (no difference was observed with longer periods of time), then centrifuged, and the contents filtered. A 0.6 ml. aliquot of the filtrate was removed and to it was added 0.4 ml. of 0.05 m sodium borate containing about 50 units of alkaline phosphatase as defined by Schmidt and Thannhauser (7).

⁴ Model 9KC magnetostriction oscillator, Raytheon Manufacturing Company, Boston, Massachusetts.

After incubation with the phosphatase for 8 hours at 38°, 0.2 ml. of 40 per cent trichloroacetic acid was added, the mixture centrifuged, and 1.0 ml. of the supernatant analyzed for inorganic phosphate.

The two procedures gave comparable results, those by the phosphatase method being somewhat more consistent. A comparison of the two methods is given in Table I.

Procedures Employed in Determining Effect of Lecithinase Action on ATPase Activity—These experiments consisted of three parts: (1) incubation of the ATPase with the lecithinase, (2) determination of ATPase activity of the mixture at the end of the incubation, and (3) denaturation of the remainder of the mixture of ATPase and lecithinase and determination of the amount of acid-soluble phosphorus formed from the ATPase by lecithinase.

The experimental arrangement consisted of mixing 1.0 ml. of the concentrated ATPase solution containing 150 to 200 γ of organic P per ml.,

Table I

Comparison of Incineration and Phosphatase Methods for Determining Lecithinase

Activity

Volume of lecithinase solution	Incineration procedure	Phosphatase procedure
ml.	γP	γΡ
0.2	29.9	34.2
0.4	69.3	65.1
The second secon	· · · · · · · · · · · · · · · · · · ·	ATTENDED TO THE PROPERTY AND ADDRESS OF THE PARTY AND ADDRESS OF THE PA

0.1 ml. of 0.075 m CaCl₂, and 0.2 ml. of the lecithinase solution. Amounts of lecithinase varied from 0.46 to 45.0 unit and the time of incubation from 10 to 60 minutes. At the end of the incubation period 0.3 ml. of the mixture was added to 4.7 ml. of cold buffer (0.06 m histidine-0.2 m KCl-0.001 m KCN). After mixing, the ATPase activity of a 0.2 ml. aliquot of this diluted material was determined. The remainder of the original ATPase-lecithinase solution was inactivated and analyzed for soluble phosphorus; both the procedures given above for the determination of lecithinase activity were used.

It was observed previously (1) that Ca inhibits the Mg-activated ATPase. However, considering the dilution of the Ca in these experiments, no significant depression of activity was anticipated. Nevertheless, to cover any instability of the ATPase during incubation at 38° for prolonged time periods under these circumstances, controls (lecithinase solution replaced by 1 per cent serum albumin) were run for each time period used. The controls always gave a small amount of soluble phosphorus (about 7 per cent of the total P) regardless of the procedure used for in-

activation and analysis. In addition, the lecithinase solutions always contained some phosphorus and corrections for this were applied.

Determination of Phospholipide Fractions of ATPase Preparations before and after Action of Lecithinase—The general plan of these experiments was to inactivate the ATPase with a large amount of lecithinase, dialyze the resulting solution to remove phosphorylcholine that had been formed, and determine the composition of the remaining phospholipide. This would then be compared with the analysis of an untreated control.

For these studies 5.0 ml. of the ATPase were mixed with 0.5 ml. of 0.075 m CaCl₂ and with about 200 units of the lecithinase dissolved in 1.0 ml. of 1 per cent serum albumin. A control with 1 per cent serum albumin substituted for the lecithinase solution was also prepared. These solutions were incubated for 60 minutes at 38° and then transferred to dialyzing tubes and dialyzed against four or five changes of histidine buffer for 16 hours in a rocking dialyzer. After dialysis, aliquots of the solutions were removed for determination of ATPase activity and for total phosphorus content. The remainder of the dialyzed solutions was lyophilized, with final drying over P₂O₅ under a vacuum. 10 mg. portions of the dried products were removed for determination of total phosphorus, and, after weighing, the remainder was extracted with a 1:1 mixture of methanol-chloroform for 12 to 20 hours.

Analyses of the distribution of lipide phosphorus were conducted according to the procedure of Hack (8). The lipide extracts were transferred to 25 ml. volumetric flasks and made up to volume with the methanol-chloroform mixture. 1.0 ml. aliquots were removed for determination of total lipide P. Of the remaining methanol-chloroform solutions, 10 ml. of the control or 20 ml. of the lecithinase-treated material were evaporated to dryness under a stream of nitrogen at 65°. The residues were then emulsified with 5.0 ml. of 1.0 N NaOH and incubated at 38° for 16 hours. At the end of this time, the solutions were cooled and to 1.0 ml. aliquots were added 1.0 ml. of 1.5 n HCl and 3.0 ml. of 10 per cent trichloroacetic acid. These were allowed to stand for 1 hour and then centrifuged and filtered. 2.0 ml. aliquots of the filtrates were then incinerated for determination of the monoaminophosphatide P. Sphingomyelin, since it is not affected by the alkali treatment, is found in the precipitate removed by centrifugation and filtration. The diaminophosphatide (sphingomyelin) P is the difference between the monoaminophosphatide P and total lipide P.

The amount of lecithin in the monoaminophosphatide fraction was obtained by choline analysis. 3.0 ml. of the alkaline solution were treated with 1.0 ml. of 4.5 n HCl. After standing for 60 minutes, the suspension was filtered through a thin layer of paper pulp with suction and the filter washed twice with 0.5 ml. portions of 1.5 n HCl. To the combined filtrates

were then added 2.0 ml. of a freshly prepared saturated solution of ammonium reineckate in 0.5 n HCl. This was allowed to stand for 30 minutes at room temperature. The crystals of choline reineckate were then collected on a Pyrex M filter and washed twice with 1.0 ml. portions of ethanol. After drying, the salt was dissolved in 3.0 ml. of acetone and the optical density determined in the Beckman spectrophotometer at 526 m μ . A standard choline curve was obtained for amounts of choline ranging from 1 to $6~\mu \text{m}$ in 5.0~ml.

Although the lecithinase preparations contained phosphate. this was all inorganic and, therefore, did not contribute to any of the fractions in these analyses.

Results

Inactivation of ATPase by Lecithinase-Fig. 1 presents the results of five experiments (Curve I) relating inactivation of the ATPase to lecithinase action. One of these (Curve II) is presented separately to indicate the uniform relationship within a single experiment. The curve in both cases is the same and was fitted to the data of the experiment which is represented separately. In these five experiments five ATPase preparations and two lecithinase preparations were employed. The characteristics of the curves are in all cases the same: an initial non-linear portion up to about 20 per cent inactivation and a linear relationship between inactivation and hvdrolvsis thereafter. Within the error of the experiment, the slopes were identical and the extrapolated maxima fell between 60 and 70 per cent hydrolysis of the total phosphorus. The values on the abscissa have been represented as per cent of the total P split off by the lecithinase, since complete data on the distribution of phosphorus were not obtained for all preparations of the ATPase. However, information on some of the preparations, such as that presented in Table IV, indicate that these extrapolated maxima represent the per cent of the total P present as lecithin and sphingomyelin. Complete inactivation of the ATPase was never attained, the maximal values lying between 75 and 90 per cent. With an incubation time of 60 minutes the maximal values were nearly obtained with about 20 units of the lecithinase; doubling the concentration of lecithinase gave only about 1 to 4 per cent increase in both inactivation and phospholipide hydrolysis.

The lecithinase preparations used in these experiments were probably not free of the other known activities of the toxic filtrates of *C. welchii*. Since the lecithinase is activated by Ca, by reducing the Ca concentration it should be possible to demonstrate whether or not the association of inactivation of the ATPase with phospholipide hydrolysis is fortuitous. Experiments of this type were performed, with and without added Ca, the

system being otherwise constant (final concentration of addition, 5.7 \times 10⁻⁸ M Ca). Some results are given in Table II. There was always some lecithinase activity in the absence of added Ca, since Ca phosphate was used in the preparation of the lecithinase and was never completely re-

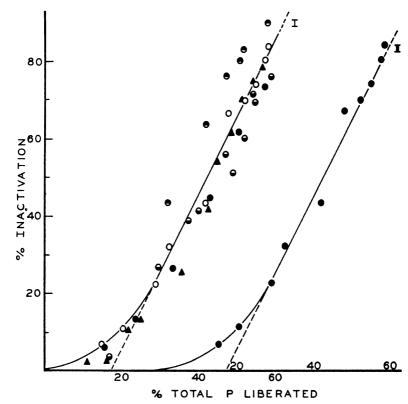


Fig. 1. Relationship between inactivation of the ATPase and formation of acid-soluble phosphorus from the ATPase preparation by lecithinase. Results for five ATPase preparations are given (Curve I). One of these (O) is presented separately to indicate the uniformity within a single experiment. The numbers on the abscissa below the line refer to the P liberated in the five experiments of Curve I; the numbers above the line refer to Curve II.

moved. Both inactivation of the ATPase and phospholipide hydrolysis were appreciably reduced in the absence of added Ca. The higher concentrations of lecithinase in Table II are in excess of that required to give maximal inactivation and hydrolysis with an optimal Ca concentration and an incubation period of 60 minutes. The lecithinase activities with and without added Ca are, therefore, not in the same proportion in all the ex-

periments. Without added Ca, the inactivation is suppressed more than the phospholipide hydrolysis. No reason for this is immediately evident, though an explanation probably resides in the complex character of the phospholipide and the relative rates with which the individual components are split by the lecithinase.

Indifference of ATPase to Products of Phospholipide Hydrolysis by Lecithinase—It seemed possible that the inactivation of the ATPase by the lecithinase might be due to inhibition by the products of hydrolysis of the phospholipides rather than to destruction of a component necessary for the activity of the ATPase. To test this an amount of lecithin corresponding

Table II

Influence of Ca on Inactivation of Mg-ATPase and on Hydrolysis of Its Phospholipide by C. welchii Lecithinase

Time,	60	minutes;	temperature	, 38°.

ATPase prepara- tion No.	Lecithinase per	Ca	added	No Ca added		
	solution	Inactivation	Hydrolysis	Inactivation	Hydrolysis	
	unsis	per cent	per cent total P	per cent	per cent total I	
9	1.3 (Prepara- tion II)	26.7	33.2	0.0	1.0	
9	17.0 (Preparation II)	73.7	57.5	8.0	26.8	
10	26.0 (Preparation III)	83.2	51.9	18.8	32.9	

to $686 \ \gamma$ of P was treated with 2 units of lecithinase and $0.2 \ \text{ml.}$ of $0.05 \ \text{m}$ CaCl₂ in a total volume of $2.6 \ \text{ml.}$ When hydrolysis was nearly complete (94 per cent), $0.5 \ \text{ml.}$ of this solution, containing $132 \ \gamma$ of P, was mixed with $0.5 \ \text{ml.}$ of a concentrated ATPase solution containing $74 \ \gamma$ of organic P. Two controls were prepared, one containing the same concentrations of Ca and lecithinase, the other a similar dilution of the ATPase with histidine buffer. After standing for 30 minutes, $0.5 \ \text{ml.}$ of each solution was diluted to $5.0 \ \text{ml.}$ with the usual histidine buffer and the ATPase activity determined on a $0.2 \ \text{ml.}$ aliquot. The results are presented in Table III. It is evident that, though the hydrolyzed lecithin constituted about 70 per cent of the total phospholipide (about 80 per cent of the ATPase phosphorus being lipide P), no inhibition of the ATPase occurred.

Composition of Phospholipide of ATPase Preparations and Changes That Occur in It through Action of Lecithinase—The results of one experiment conducted according to the procedure given earlier for determining lipide P distribution are presented in Table IV. In this experiment 90 per cent of the ATPase activity was lost as a result of the lecithinase treatment. As a check on the effectiveness of the dialysis employed in this analysis, a parallel experiment was carried out on a smaller scale with the same material but with immediate analysis at the end of the incubation period. In this case, the amount of phospholipide split was determined by the

Table III

Indifference of Mg-ATPase to Products of Phospholipide Hydrolysis by C. welchii

Lecithinase

Hydrolyzed lecithin	Lecithinase	ATPase activity in 5 min.		
	THE WASTERS TO CONTRACT AND ADMINISTRATION OF THE PARTY O	γ P		
	_	58.2		
	+	57.6		
+	+	57.6		
i				

TABLE IV

Distribution of Organic P in ATPase before and after Treatment with Lecithinase

The results are expressed in micromoles of P per ml

	Control ATPase solution (1)	Lecithinase- treated ATPase solution (2)	Difference, ATPase solution (1) - (2)
Total P	5.05	1 93	3.12
" lipide P	4 07	1.12	2.95
Monoaminophosphatide P	3.65	0 98	2.67
('holine (lecithin)	2.69	0.06	2.63
Sphingomyelin P	0.42	0.14	0.28

phosphatase procedure. Identical values were obtained for loss of activity and change in total bound P in the two experiments.

Within the probable error involved in the analyses presented in Table IV, the change in total P is accounted for by the change in lipide P. There appears to be no contribution from the cephalin fraction (monoamino-phosphatide P minus lecithin P) and the change in lipide P is equal to the sum of the changes in lecithin (choline) and sphingomyelin which together comprise the "choline phosphatide fraction."

DISCUSSION

Except for the initial non-linear portion of the curves in Fig. 1, inactivation of the ATPase and hydrolysis of phospholipide exhibited strictly

parallel courses. Maximal values appear to be a characteristic of the individual ATPase preparation. These maxima were not significantly increased by prolonging the time of incubation and increasing the amount of lecithinase. Inhibition of the lecithinase by the cephalin fraction when hydrolysis of the choline-containing phosphatides has approached completion probably accounts for the fact that complete inactivation of the ATPase was never attained. Zamecnik et al. (4) have demonstrated that "cephalin" will inhibit the lecithinase and by examining Table IV it is observed that, whereas the initial concentrations of the "cephalin" and choline phosphatide (lecithin + sphingomyelin) fractions were 0.96 and 3.11 µm per ml. respectively, at the end of the experiment they are 0.92 and 0.20 µm. In addition, the hydrolyzable substrate at the end of the experiment is primarily sphingomyelin which, as demonstrated by Macfarlane (5), is attacked at a slower rate than lecithin. Thus the changing relative concentrations of the phospholipide components have combined with the actual change in substrate concentration, in any experiment in which either time or lecithinase concentration was varied, to slow down and finally halt phospholipide hydrolysis and with it the inactivation of the ATPase.

The dependence of inactivation of the ATPase on phospholipide hydrolysis is further substantiated by the control exerted by Ca concentration over both phenomena.

With the demonstration that the products of lecithinase action do not suppress the ATPase activity, the conclusion seems justified that a phospholipide component of the partially purified Mg-ATPase of muscle is essential for the characteristic enzymatic activity of these preparations.

The ATPase preparations analyzed in this investigation contained all three of the general classes of phospholipides and of these both lecithin and sphingomyelin were split by the lecithinase; therefore, we can only state that the essential component is a choline-containing phospholipide.

SUMMARY

The inactivation of partially purified preparations of the magnesium-activated ATPase of muscle by *Clostridium welchii* lecithinase has been studied.

It has been found that the inactivation of the ATPase depends on the lecithinase activity and that, over the upper 80 per cent of the range, the splitting of phospholipide and inactivation are proportional.

It is concluded that a choline-containing component of the phospholipides present in the ATPase preparation is essential for the characteristic enzymatic activity.

BIBLIOGRAPHY

- 1. Kielley, W. W., and Meyerhof, O., J. Biol. Chem., 176, 591 (1948).
- 2. van Heyningen, W. E., Biochem. J., 35, 1257 (1941).
- 3. Macfarlane, M. G., and Knight, B. C. J. G., Biochem. J., 35, 844 (1941).
- 4. Zamecnik, P. C., Brewster, L. E., and Lipmann, F., J. Exp. Med., 85, 381 (1947).
- 5. Macfarlane, M. G., Biochem. J., 42, 587 (1948).
- 6. Levene, P. A., and Rolf, I. P., J. Biol. Chem., 72, 587 (1927).
- 7. Schmidt, G., and Thannhauser, S. J., J. Biol. Chem., 149, 369 (1943).
- 8. Hack, M. H., J. Biol. Chem., 169, 137 (1947).

A COLORIMETRIC METHOD FOR THE DETERMINATION OF CHYMOTRYPSIN ACTIVITY*

By BEAT M. ISELIN,† H. T. HUANG, AND CARL NIEMANN

(From the Gates and Crellin Laboratories of Chemistry ‡ California Institute of Technology, Pasadena)

(Received for publication, October 31, 1949)

The L isomers of esters, amides, and hydrazides of the general formula RCONHCHR₁COR₂ where $R = CH_3$ —, C_6H_6 —, etc., $R_1 = C_6H_6CH_2$ —, p-HO— $C_6H_4CH_2$ —, etc., and $R_2 = -OCH_3$, —NH₂, or —NHNH₂, are known to be hydrolyzed by chymotrypsin (1–6). It has now been found that the corresponding hydroxamides, RCONHCHR₁CONHOH, where $R_1 = C_6H_6CH_2$ —, are also hydrolyzed by this enzyme (Table I). Aside from the obvious usefulness of the above hydroxamides in the further definition of chymotrypsin activity we wish to point out that these latter substrates provide the basis for a simple, sensitive, and rapid colorimetric method for the determination of chymotrypsin activity which can be extended to a number of other proteolytic enzymes.

It is well known that acylhydroxamides, i.e. hydroxamic acids, react with ferric ion, in acidic solutions, to give characteristic deep red colored coordination compounds (7) and it has been found that the above reaction can be used in a quantitative way to determine the rate of disappearance of hydroxamides of the general formula, RCONHCHR₁CONHOH, when these latter compounds are being hydrolyzed by a proteolytic enzyme, in this case chymotrypsin. In practice an aliquot portion of the enzymatic reaction mixture is added to a standard acidic aqueous-methanol solution of ferric chloride, the intensity of the color is observed in a photoelectric colorimeter, and the amount of hydroxamic acid present is estimated from a previously determined calibration curve.

In addition to their offering the basis for a colorimetric procedure the hydroxamides of the general formula RCONHCHR₁CONHOH possess other characteristics that make them desirable substrates. For compounds of the type RCONHCHR₁COR₂, where $R = CH_3$ — or C_6H_5 — and $R_1 = C_6H_5$ CH₂—, the rate of hydrolysis, by chymotrypsin, of the corresponding esters, amides, and a glroxamides, i.e $R_2 = -OCH_3$, $-NH_2$, and -NHOH when R and R_1 are invariant, is in the order ester > hydroxamide >

^{*} Supported in 1 art by a grant from Eli Lilly and Company.

[†] Present address, The Squibb Institute for Medical Research, New Brunswick, New Jersey.

[‡] Contribution No. 1348.

amide (4, 8). Thus the hydroxamides are hydrolyzed sufficiently rapidly to permit their use with relatively low enzyme concentrations and to allow the attainment of saturation of the enzyme at relatively low substrate concentrations. These factors, coupled with the greater sensitivity of the colorimetric method, make it possible to study the hydrolytic reaction at initial substrate concentrations of less than 2.5 micromoles per ml. with a greater precision than is practicable with titrimetric methods. A further desirable characteristic of the hydroxamide type of substrate is based upon the fact that these compounds are acids and, even though their acid

Table I

Hydrolysis of Acetyl- and Benzoyl-L-phenylalaninhydroxamides by Chymotrypsin*

Substrate	Sot	E ₀ ‡	Buffer concen- tration§	Analytical method	Per cent hydrolysis in		
Substrace					20 min.	40 min.	60 min.
Acetyl-DL	5	0.15	0.04	A	34	53	69
44	5	0.15	0.04	В	35	52	67
46	2.5	0.15	0.02	A	40	69	84
"	2.5	0.15	0.02	В	40	70	82
Benzoyl-DL	2.5	0.15	0.02	A	Ca. 8	1 80% in	7 min.
ii	2.5	0.03	0.02	"	58	76	80

^{*} At 25° and pH 7.9 (tris(hydroxymethyl)aminomethane-hydrochloric acid buffer).

strength is not great (7), it is possible to use the corresponding salts with those enzymes whose pH optima are greater than 7 in the event that the hydroxamides per se are relatively insoluble in water. It should be pointed out that if this latter procedure is adopted, and particularly if an alkali metal salt is used, care must be taken to provide a system of adequate buffering capacity.

The advantages of the above colorimetric method for the determination of proteolytic activity over other colorimetric methods (9, 10) are obvious when it is realized that the former method is based upon the use of specific substrates of known structure, whereas the latter are dependent upon relatively non-specific substrates of unknown structure.

[†] Initial concentration of L isomer in micromoles per ml. of reaction mixture.

[‡] Initial enzyme concentration in mg. of protein nitrogen per ml. of reaction mixture

[§] Molar concentration of tris(hydroxymethyl)aminomethane in reaction mixture.

As sodium salt.

In the present study the DL-hydroxamides have been used as substrates. In view of the fact that it is known that in other cases the D isomer can competitively inhibit the hydrolysis of the L isomer and that this effect is maximal at apparent enzyme saturation (11), it is recommended that in the application of the above colorimetric method the pure L isomer be used as a substrate until sufficient data are available to evaluate the magnitude of the various rate and inhibition constants. Such studies are now in progress.

EXPERIMENTAL¹

Acetyl-denylalaninhydroxamide—To a solution of 12.5 gm. of 2-methyl-4-benzyl-5-oxazolone, b.p. 115-116° at 1 mm. (12) in 15 ml. of anhydrous ether, were added, with vigorous shaking and cooling in an ice-salt bath, 65.5 ml. of a 5 per cent anhydrous methanol solution of hydroxylamine. The reaction mixture was allowed to stand overnight at room temperature, filtered, the filtrate evaporated to dryness, the syrupy residue extracted with four 225 ml. portions of hot ethyl acetate, and the filtered ethyl acetate extract concentrated in vacuo to 150 ml. After standing for 20 hours, the last three at 0°, the crystalline precipitate was collected and dried to give 8.17 gm. of acetyl-denylalaninhydroxamide, m.p. 120-125°, with softening at 110°. After two recrystallizations from ethyl acetate, the product melted at 131-132° with decomposition.

Benzoyl-dl-phenylalaninhydroxamide—To a solution of 14 gm. of benzoyl-dl-phenylalanine methyl ester in 50 ml. of anhydrous methanol were added 24.5 ml. of a 10 per cent methanol solution of hydroxylamine. The solution was cooled to 0°, 49.4 ml. of n methanolic sodium methoxide were added, and the reaction mixture allowed to stand at room temperature for 48 hours. The precipitated solid was dissolved by warming the solution to 40°. The solution was cooled to 0°, acidified to Congo red with 6 n hydrochloric acid, evaporated to dryness in vacuo, and the residue dried over solid sodium hydroxide and extracted with a total of 700 ml. of hot ethyl acetate. The extract was cooled, and the crystalline precipitate collected and dried to give 10.8 gm. of benzoyl-dl-phenylalaninhydroxamide, m.p. 157–158° with decomposition. A second crop of 1.2 gm., m.p. 156–157° with decomposition, was obtained from the mother liquor. A portion of the above product was recrystallized from n-butanol

¹ All the melting points are corrected.

to give the hydroxamide, m.p. 158-159° with decomposition and with preliminary softening at 152°.

Method A—A 1.0 ml. aliquot of the enzymatic digest was added to 1.0 ml. of 36 per cent aqueous formaldehyde, previously adjusted to pH 7.0, and the solution titrated potentiometrically with 0.01 N aqueous sodium hydroxide (13).

Method B—A standard ferric chloride solution was prepared as follows: 0.50 gm. of reagent grade anhydrous ferric chloride was dissolved in 80 ml. of 1.0 N hydrochloric acid and the volume of the solution made up to 100 ml. with absolute methanol. A 1.0 ml. aliquot of the enzymatic digest was added to 5.0 ml. of absolute methanol and 1.0 ml. of standard ferric chloride solution, contained in a calibrated colorimeter tube, and the volume of the solution made up to 10.0 ml. by the addition of water. The contents of the tube were shaken and the intensity of the color determined with a Klett-Summerson photoelectric colorimeter equipped with a green filter. Solutions containing varying amounts of substrate in either 0.02 m or 0.04 m tris(hydroxymethyl)aminomethane-hydrochloric acid buffer were used for the preparation of a standard calibration curve. values observed for the two buffer concentrations were identical and it was found that for concentrations of hydroxamide between the limits of 1 and 10 micromoles per ml. of original solution the colorimeter readings were directly proportional to the concentration of hydroxamide. presence of enzyme in concentrations up to 0.15 mg. of protein nitrogen per ml. of original solution had no demonstrable effect upon the intensity of the color at any given hydroxamide concentration. However, at high concentrations of hydroxamide it was observed that the intensity of the color slowly faded on standing and because of this phenomenon the time interval between the time of mixing and the time of reading was standardized at 90 seconds. The experimental conditions specified for the color development were selected with regard to their suitability for the attainment of the following objectives; i.e., immediate cessation of enzymatic activity, avoidance of the precipitation of substrate or hydrolysis products, and the development of maximal color intensity.

Enzyme Experiments—The chymotrypsin was an Armour preparation and the data obtained are given in Table I. The conventional controls, i.e. substrate alone and enzyme alone, were performed coincidentally with each separate experiment.

SUMMARY

It has been observed that the acetyl- and benzoyl-L-phenylalaninhy-droxamides are hydrolyzed by chymotrypsin, and a colorimetric method for the determination of chymotrypsin activity based upon the use of hydroxamides as substrates has been described.

BIBLIOGRAPHY

- 1. Bergmann, M., and Fruton, J. S., J. Biol. Chem., 118, 405 (1937).
- 2. Fruton, J. S., and Bergmann, M., J. Biol. Chem., 145, 253 (1942).
- 3. Kaufman, S., Neurath, H., and Schwert, G. W., J. Biol. Chem., 177, 793 (1949).
- 4. Snoke, J. E., and Neurath, H., Arch. Biochem., 21, 351 (1949).
- 5 Kaufman, S., and Neurath, H., Arch. Biochem., 21, 437 (1949).
- 6. MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc., 71, 3854 (1949).
- Sidgwick, N. V., The organic chemistry of nitrogen, edited by Taylor, T. W. J., and Baker, W., Oxford (1937).
- 8. Iselin, B. M., Huang, H. T., MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc., in press.
- 9. Anson, M. L., J. Gen. Physiol., 22, 79 (1938).
- Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 287 (1937).
- MacAllister, R. V., Harmon, K. M., and Niemann, C., J. Biol. Chem., 177, 767 (1949).
- 12. Bergmann, M., Stern, F., and Witte, C., Ann. Chem., 449, 277 (1926).
- 13. Iselin, B. M., and Niemann, C., J. Biol. Chem., 182, 821 (1950).

THE NON-ENZYMATIC REDUCTION OF CYTOCHROME c BY PYRIDINE NUCLEOTIDES AND ITS CATALYSIS BY VARIOUS FLAVINS*

BY THOMAS P. SINGER AND EDNA B. KEARNEY†

(From the Department of Biochemistry, School of Medicine, Western Reserve
University, Cleveland)

(Received for publication, August 18, 1949)

It is now generally accepted that a large fraction of cellular oxidations is mediated by the pyridine nucleotide-flavoprotein-cytochrome-cytochrome oxidase system. Since the isolation of the first flavoprotein by Warburg and Christian (1), several flavoenzymes have been discovered. Although some of these are concerned with the direct oxidation of various metabolites (e.g., the amino acid oxidases) and others (e.g., cytochrome reductases. diaphorases) mediate electron transfer from reduced pyridine nucleotides to cytochrome and other oxidizing agents, the most important known rôle of flavoproteins in biological oxidations, according to Ball (2), remains "their participation as mediators in oxidations initiated by means of the pyridine nucleotides." While it is generally recognized that the isoalloxazine nucleus of the prosthetic group is the functional unit of flavoenzymes. to the authors' knowledge it has not been demonstrated that isoalloxazine derivatives themselves, in the absence of added proteins, are capable of catalyzing the reaction between reduced pyridine nucleotides and cytochrome c.

In the course of a study¹ of the inhibition of cytochrome c reductase (TPN, yeast) (3) by isoriboflavin we observed that in the presence of isoriboflavin or riboflavin cytochrome was rapidly reduced by TPNH₂ despite the absence of cytochrome reductase. We decided to investigate this reaction in detail. This paper demonstrates the reduction of cytochrome c by reduced DPN, TPN, and NMN and the catalytic rôle of various flavins in the reaction.

Materials and Methods

Flavins—Riboflavin was purchased from Wyeth, Inc., and was found to be a pure compound within the limits of the spectrophotometric method.

- * This research was supported by a grant from The National Vitamin Foundation, Inc.
 - † Senior Research Fellow of the National Cancer Institute.
- ¹ The following abbreviations are used: TPN, triphosphopyridine nucleotide; TPNH₂, reduced TPN; DPN, diphosphopyridine nucleotide; DPNH₂, reduced DPN; NMN, nicotinamide mononucleotide; NMNH₂, reduced NMN; FAD, flavin-adenine dinucleotide; FM, flavin mononucleotide.

The isoriboflavin was a gift of Dr. W. Gibson of Merck and Company and was analytically pure as indicated by C, H, and N analysis. Alloxazine was obtained from the Bios Laboratories, Inc. Flavin-adenine dinucleotide (FAD) was isolated from bakers' yeast by the procedure of Warburg and Christian (4); its concentration was measured by the light absorption at 445 m μ and by the p-amino acid oxidase test (4). Flavin mononucleotide (FM) was synthesized from riboflavin (5) and its concentration was determined spectrophotometrically and by phosphorus analysis. All flavin solutions were protected from light as much as possible.

Cytochromes²—Two preparations of cytochrome c were used in most of the experiments, one from Wyeth, Inc., and the other from the Nutritional Biochemicals Corporation. Certain of the key experiments were repeated with a cytochrome of high purity (0.38 per cent Fe), prepared according to the method of Keilin and Hartree (6) and kindly provided by Dr. Ernest Bueding of the Department of Pharmacology. Purity of the cytochromes was estimated by measuring the ratio of the extinction coefficients of the oxidized and reduced forms, at 550 mµ, after treatment with ferricyanide and Na hydrosulfite, respectively (7). The ratio obtained (0.329 to 0.330) was in good agreement with the figures given by Potter (7) and by Theorell (8). The concentration of cytochrome solutions was calculated from the light absorption at 550 mµ following reduction by hydrosulfite, by use of the relation CyFe⁺⁺ = $(D/0.281) \times 10^{-8}$ mole per ml., when D = the optical density in a 1 cm. path (3). In all measurements of cytochrome reduction the Beckman spectrophotometer was used, and the amount of CyFe+++ present at any time was calculated from the optical density at 550 m μ by means of the equation developed by Haas et al. (3): CyFe⁺⁺⁺ = $(1/l) \times D - 0.281 \times 10^8 \times (\text{total CyFe})/(-0.1854 \times 10^8)$, where l =the length of the light path in cm., D = the optical density directly read on the spectrophotometer, and cytochrome concentrations are expressed in moles per ml.

Pyridine Nucleotides—Two preparations of TPN were used, one of 37 per cent and the other of 51 per cent purity, as determined by enzymatic analysis in the Zwischenferment test (9). We are grateful to Dr. S. Ochoa, of New York University, for these samples of TPN. DPN (Schwarz Laboratories, Inc.) was estimated spectrophotometrically at 340 m μ following hydrosulfite or enzymatic reduction, and varied in purity from 50 to 60 per cent. Solutions of DPNH₂ were prepared by Gutcho and Stewart's modification of the hydrosulfite method (10), except that the final product was made 1.5×10^{-2} M with respect to phosphate buffer and the pH was

² The terms ferricytochrome c, oxidized cytochrome c, and CyFe⁺⁺⁺ are used interchangeably in this paper, and similarly for the reduced forms.

² Only the presence of colored impurities is detected by this method.

adjusted to 7.6 to 7.9, depending on the experiment. For reasons discussed in the paper, solutions of DPNH₂ thus prepared were either used immediately or were preserved for short periods of time, preferably at -40° . The concentration of DPNH₂ solutions was calculated from the molecular extinction coefficient at 340 m μ (11), and their true DPNH₂ content was frequently checked by oxidation with acetaldehyde in the presence of alcohol oxidase. Some preparations of DPNH₂ were subjected to alcohol fractionation (11) in order to obtain a dry product free from inorganic salts; the purity was thereby raised to 78 per cent. Solutions of NMNH₂ were prepared from DPNH₂ by the action of nucleotide pyrophosphatase (12), kindly supplied by Dr. A. Kornberg of the National Institutes of Health. Complete splitting of DPNH₂ into NMNH₂ was ascertained by its lack of activity in the alcohol oxidase system, although the light absorption at 340 m μ was unaltered.

Enzymes—Zwischenferment was prepared from brewers' bottom yeast (1). The phosphoglyceraldehyde oxidase from yeast was a lyophilized preparation of the partially purified enzyme, made by Miss Aeme Higashi, of the Department of Pharmacology, by the procedure of Meyerhof and Junowicz-Kocholaty (13). Phosphoglyceraldehyde oxidase from rabbit muscle was prepared according to Cori, Slein, and Cori (14), and was used as the once recrystallized enzyme. Alcohol oxidase was isolated from yeast and assayed by the procedure of Negelein and Wulff (15); the purification was carried through the second (NH₄)₂SO₄ precipitation. A lyophilized preparation of cytochrome oxidase was prepared by Mr. T. Talcott of this department by an unpublished modification of the Keilin and Hartree procedure (16).

EXPERIMENTAL

Reduction of Cytochrome by Enzymatically Prepared $TPNH_2$ —The protocol and results of the first experiment which led to the observation of the non-enzymatic reduction of cytochrome c are presented in Table I. Since this experiment was intended to measure the effect of riboflavin on cytochrome c reductase, the experimental conditions conform approximately with those used by Haas $et\ al$. for the assay of that enzyme (3). All components except riboflavin were incubated for 10 minutes at 25° to permit the reduction of TPN by the glucose-6-phosphate-Zwischenferment system. Riboflavin was added at zero time and readings were taken every minute against blanks which contained all additions except cytochrome c. It will be seen from Table I that in the complete system 20 per cent of the added cytochrome was reduced in 6 minutes at 25°. When either glucose-6-phosphate or riboflavin was omitted, no significant reduction of cytochrome occurred. In parallel experiments with the same amount of isoriboflavin

 $(4 \times 10^{-7} \text{ mole per ml.})$ cytochrome c was reduced over twice as fast as in the experiment above.

There could be three possible interpretations of these findings. First, riboflavin and isoriboflavin may have catalyzed the interaction of TPNH₂ with ferricytochrome c, replacing cytochrome c reductase; second, an impurity in one of the reagents may have performed this function; or third, the Zwischenferment may have contained some split reductase protein (apoenzyme) with which the added flavins united to form an active reductase complex. The third possibility would require a considerable lack

TABLE I
Reduction of Ferricytochrome c in Presence of Riboflavin

	D at 550 mu, read vs. reagent blank				
No glucose-6- phosphate	No riboflavin	Complete system	Time		
		.,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	min.		
0.462	0.449	(0.462)	0		
0.463	0.448	0.505	1		
0.465	0.449	0.522	2		
0.466	0.449	0.546	3		
0.467	0.448	0.556	4		
0.468	0.449	0.583	5		
0.468	0.449	0.601	6		
		3.73×10^{-8} mole per ml.	$(CyFe^{+++}) \text{ at } t = 0$		
1		2.99 × 10 ⁻⁸ " " "	" " = 6 min.		
		0.74 × 10 ⁻⁸ " " "	$\Delta(\text{CyFe}^{+++})$ in 6 min.		

Zwischenferment, 1 ml. ≈ 0.8 mg. in 0.1 M PO₄, pH 7.3, 0.4 ml.; glucose-6-phosphate, 1 ml. ≈ 6.1 mg. of Ca salt, 0.4 ml.; TPN, 1 ml. $\approx 810 \gamma$ of 37 per cent pure substance, 0.2 ml.; PO₄ buffer, 0.1 M, pH 7.3, 0.35 ml.; CyFe⁺⁺⁺, 2.5 × 10⁻⁴ M, 0.45 ml.; riboflavin, 2 × 10⁻³ M in 5 × 10⁻⁸ M NaOH, 0.6 ml.; H₂O to 3.0 ml.; pH 7.44.

of specificity in the requirements of the prosthetic group of cytochrome reductase, but none the less was tested in two ways designed to inactivate any reductase present. First, the Zwischenferment system was used to accumulate TPNH₂ as before, with corresponding blanks lacking glucose-6-phosphate. These solutions were then cautiously acidified to pH 2.5 to 3.0 at 0° with 20 per cent CCl₃COOH and rapidly filtered in the cold. The neutralized filtrates gave negative biuret reactions and were devoid of Zwischenferment activity in the Haas dye test (9). The second means of inactivating the Zwischenferment system following accumulation of TPNH₂ was by heating the solutions in a boiling water bath for 2 minutes, and rapidly cooling them to room temperature. Although both of these tech-

niques inactivated much of the TPNH₂ present, the light absorption at 340 m μ indicated enough TPNH₂ to permit the use of these two preparations as reducing agents for cytochrome c in the presence of riboflavin. With both preparations there was a rapid and continuous reduction of cytochrome until the TPNH₂ was exhausted. No reduction occurred with the corresponding blanks prepared without glucose-6-phosphate. These findings made it unlikely that a cytochrome reductase impurity in the Zwischenferment was responsible for the reduction of cytochrome in the original experiment. It should be added, however, that a preparation of Zwischenferment essentially free from reductase, kindly placed at our disposal by Dr. B. L. Horecker of the National Institutes of Health, gave entirely similar results.

The second possibility, that impurities in the substances used were reducing cytochrome, was next studied. Since the glucose-6-phosphate, riboflavin, and isoriboflavin were analytically pure compounds, such impurities would be most reasonably expected in the *Zwischenferment*, cytochrome, or TPN. In order to eliminate these factors in *Zwischenferment* and in TPN, it was thought advisable to establish the reduction of cytochrome by DPNH₂, prepared either by hydrosulfite reduction or by enzymatic reduction of DPN.

Reduction of Cytochrome by Non-Enzymatically Reduced DPN-The experiment illustrated in Fig. 1 demonstrates the oxidation of chemically reduced DPN by ferricytochrome c, as catalyzed by various amounts of ribo-The DPNH₂ was prepared, as described above, immediately before the experiment. A control solution of hydrosulfite in bicarbonate-carbonate buffer, but without DPN, was carried through the Gutcho and Stewart procedure and then treated with riboflavin and cytochrome. No reduction of the latter occurred, indicating the complete removal of reducing substances other than DPNH2 itself by the aeration procedure. It is apparent from Fig. 1 that the chemically reduced DPN preparation, like the enzymatically reduced TPN described above, is capable of reducing ferricytochrome c in the presence of riboflavin, but not in its absence. and that the rate of the reaction is approximately proportional to the concentration of riboflavin. It is also apparent that the rate of reduction of cytochrome c at all flavin concentrations follows a second order reaction. wherein two reactants (2CyFe+++), present at equal concentrations, determine the rate. This conclusion was arrived at by plotting (CyFe⁺⁺⁺), $\log (CvFe^{+++})$, and $1/(CvFe^{+++})$ as a function of time. Only the last one gave a linear relationship. It may be noted that the data presented in Table I also follow a second order reaction curve. The significance and interpretation of these reaction orders will be discussed in the section on Similar results were obtained when the DPNH₂ preparation was kinetics.

further purified and freed of inorganic salts by fractional alcohol precipitation (11).

Reaction of Enzymatically Reduced DPN with Ferricytochrome c—In order to substantiate the fact that the DPNH₂ itself, rather than an impurity, was the reducing agent in the foregoing experiments, DPN was reduced

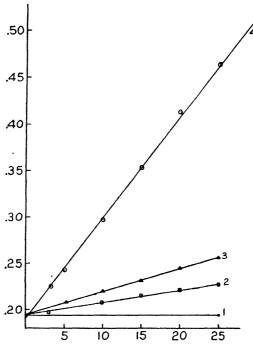


Fig. 1. The rate of reduction of ferricytochrome c by DPNH₂ at various flavin concentrations. Abscissa, time in minutes; ordinate, $1/(\text{CyFe}^{+++})$ moles per ml. \times 10^{-8} . Curve 1, no riboflavin; Curves 2, 3, and 4, 3.4×10^{-8} , 8.6×10^{-8} , and 34.3×10^{-8} mole per ml. of riboflavin, respectively. Each cuvette contained in a volume of 3.5 ml. 5.15×10^{-8} mole per ml. of CyFe⁺⁺⁺, 1 ml. of 0.1 m phosphate, pH 7.3, 6.31 \times 10^{-8} mole per ml. of DPNH₂, and the indicated amount of riboflavin. Average temperature, 26.3°; readings taken at 550 m μ .

specifically by means of three enzymes, and the activity of the resulting products on cytochrome c and riboflavin was subsequently determined. The first enzyme used was alcohol oxidase from yeast.

Dimedon (dimethyldihydroxyresorcinol) was used as an aldehyde fixative in the reaction, since it does not react with cytochrome or inhibit alcohol oxidase, but efficiently binds acetaldehyde. In some experiments

alcohol oxidase was inactivated by means of p-chloromercuribenzoate, after complete reduction of the DPN, before the addition of cytochrome and riboflavin to the system. The results were the same whether this procedure was followed or the DPNH₂ was continuously regenerated by the active oxidase throughout the subsequent reaction with riboflavin and cytochrome.

Fig. 2 summarizes the results of a typical experiment on the reaction between ferricytochrome c and DPNH₂ produced by the action of alcohol oxidase. It is seen that no reduction took place when alcohol was omitted and the accumulation of DPNH₂ was thereby prevented (Curve 3); very little reaction took place without added flavin (Curve 1), while riboflavin in a final concentration of 24.2×10^{-8} mole per ml. brought about a rapid reduction of the cytochrome (Curve 2). The concentration of CyFe⁺⁺⁺ at given time intervals is plotted directly in Fig. 2, rather than its reciprocal since the reaction did not follow strictly a second order reaction curve.

Entirely similar results were obtained when DPNH₂ was produced by means of crystalline phosphoglyceraldehyde oxidase from rabbit muscle or by a highly purified preparation of this enzyme from yeast, except that the aerobic reduction of cytochrome by the DPNH₂ thus obtained followed a second order reaction rate.

Inasmuch as the reduction of cytochrome depended on the presence of $\mathrm{DPNH_2}$ or $\mathrm{TPNH_2}$ and flavin in all of the foregoing experiments, regardless of the means by which the reduced pyridine nucleotides were generated, it seemed logical to conclude that these agents themselves were necessary in this reaction. There remained two points to be tested: (a) whether a colored impurity in the cytochrome preparation or ferricytochrome c itself was the oxidizing agent; (b) whether an impurity present in the cytochrome in small amounts acted as a catalyst for the oxidation-reduction reaction. The first of these points was eliminated in part by the purity measurements summarized under "Materials and methods," but was further tested as described in the following section.

Reduction of Cytochrome from Various Sources, Effect of Cytochrome Oxidase—Five different cytochrome c preparations were tested on DPNH₂ as substrate under the conditions given in Fig. 1. These were products obtained from Wyeth, Inc., Sharp and Dohme, Inc., the Nutritional Biochemicals Corporation, and the Sigma Chemical Company. The fifth product was the Keilin and Hartree preparation, containing 0.38 per cent Fe. All of these cytochromes behaved in an identical manner. In addition, it should be mentioned that the oxidized cytochrome could be completely reduced, particularly under anaerobic conditions, provided that enough reduced nucleotide was present. Lastly, the experiment recorded

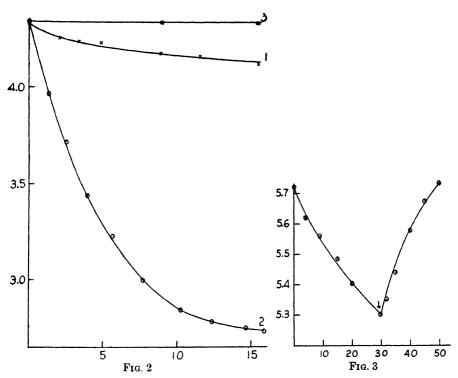


Fig. 2. Reduction of cytochrome c with and without added riboflavin with continuous production of DPNH₂ by alcohol oxidase. Abscissa, time in minutes; ordinate, CyFe⁺⁺⁺ concentration in moles per ml. \times 10⁻⁸. Curve 1, no flavin added; Curve 2, 24.2 \times 10⁻⁸ mole per ml. of riboflavin; Curve 3, no alcohol. Two identical tubes containing 0.4 ml. \approx 0.96 mg. of 50 per cent DPN, 1.2 ml. of 10⁻¹ m pyrophosphate, pH 7.8, 0.1 ml. of 3 per cent glycine, 0.3 ml. of 5×10^{-2} m dimedon, 0.3 ml. of 3 m ethanol, and 0.1 ml. of 1:150 alcohol oxidase were incubated for 10 minutes at 30°. Tube 1 received 0.5 ml. of 2.87×10^{-4} m cytochrome + 0.4 ml. of H₂O (Curve 1). To Tube 2, 0.5 ml. of cytochrome + 0.4 ml. of 2×10^{-3} m riboflavin were added (Curve 2). The blank was in every respect identical with Tube 2, except that no alcohol was present during the preliminary incubation (Curve 3). Temperature, 30°; pH 8.12.

Fig. 3. Reduction of cytochrome c by DPNH₂ and riboflavin and subsequent reoxidation by cytochrome oxidase. Abscissa, time in minutes; ordinate, CyFe⁺⁺⁺ concentration in moles per ml. \times 10⁻⁸. Reaction mixture, 1 ml. of 10⁻¹ m phosphate, pH 7.3, 0.6 ml. of 3.65 \times 10⁻⁴ m DPNH₂ (reduced by hydrosulfite), 0.6 ml. of 2 \times 10⁻⁸ m riboflavin, 0.45 ml. of 4.43 \times 10⁻⁴ m Wyeth cytochrome, and 0.85 ml. of H₂O. After 30 minutes at 30°, 0.03 ml. containing 0.03 mg. of cytochrome oxidase preparation was added at the point indicated by the arrow.

in Fig. 3 shows that after partial reduction of the cytochrome by $DPNH_2$ and riboflavin the ferrocytochrome c could be completely reoxidized by cytochrome oxidase.

These observations demonstrate that cytochrome c itself is the oxidizing agent in the system. The fact that various cytochrome preparations behave identically also witnesses against the presence of a catalytic impurity.⁴ The reaction involving the three components DPNH₂ or TPNH₂, riboflavin, and ferricytochrome c may then be postulated as follows:

$$DPNH_2 \text{ or } TPNH_2 + flavin \rightarrow DPN \text{ or } TPN + flavin H_2$$
 (1)

Flavin
$$H_2 + 2CyFe^{+++} \rightarrow flavin + 2CyFe^{++} + 2H^+$$
 (2)

The next two sections deal with the study of the two individual steps in the over-all reaction.

Reduction of Ferricytochrome c by Leucoflavin—A small amount of riboflavin (about $0.2~\mu\text{M}$) in phosphate buffer, pH 7.3, was placed in the main compartment of a Thunberg tube, equipped with gas inlet and outlet, along with a trace of Pd black, and a solution of ferricytochrome was placed in the bulb of the tube. H₂ was then passed through the tube until the riboflavin was bleached (about 15 minutes at 30°), at which time the cytochrome was tipped in. Immediate examination with a small hand spectroscope revealed the presence of the two characteristic bands of reduced cytochrome c. In the corresponding control, which contained no riboflavin, the reduced cytochrome bands did not appear. This seemed to offer a possibility for the quantitative study of the second part of the over-all reaction; i.e., the reduction of cytochrome by leucoflavin.

For the quantitative measurement of this reaction a special tube (Fig. 4) was designed to fit the Beckman spectrophotometer, which provided strictly anaerobic conditions to prevent autoxidation of the leucoflavin, and at the same time provided a means of removing the Pd prior to addition of the leucoflavin to the cytochrome. In general the riboflavin and Pd black were placed on the sintered funnel, protected from light, and the other constituents, including cytochrome, were in the main tube. All connections were sealed with paraffin. A slow stream of H₂ was bubbled through the solution in the main tube for a few minutes, under constant shaking; then the gas inlet (e) was raised and a steady stream of H₂ was passed for 30 minutes over the solution and up through the sintered funnel. When all of the leucoflavin was reduced, the flow of gas was reversed by means of a 3-way stop-cock, and the H₂ pressure, plus a small vacuum

⁴ The demonstration that the first of these two reactions will occur without cytochrome eliminates the possibility of a catalytic impurity in the cytochrome preparations.

 $^{^{5}}$ Although Theorell has shown that Pt black suspended directly in cytochrome will bring about a slow reduction if $\rm H_{2}$ is bubbled through the solution (17), quantitative determinations indicated that with Pd black, particularly in our set-up, the reduction of cytochrome was negligible for at least 15 minutes.

applied at g, forced the reduced flavin through the sintered plate into the cytochrome solution in the main tube. The tube was then disconnected, and spectrophotometric readings were begun. Between readings the tube was kept in a constant temperature bath at 30°. In order to allow the tube to fit snugly into the Beckman cuvette carrier, small adapters were made from brass cork borers, with windows for light passage and a wedge soldered on each to prevent rotation in the cuvette carrier (Fig. 4). The top of the cell compartment was covered with black sateen cloth to prevent the entry of light during readings. Each tube was calibrated with a

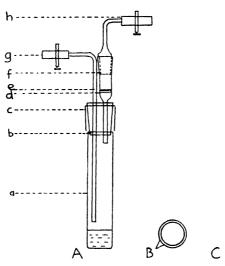


Fig. 4. Apparatus for anaerobic measurements. A, anaerobic tube; B and C, brass adapter, in cross-sectional and frontal view; a, Trubore test-tube 11 mm. inside diameter, 141 mm. long; b, 2-hole rubber stopper, No. 0; c, 14/20 outer joint; d, 2 ml. fine sintered glass funnel, containing Pd and the solution to be reduced; e, movable gas inlet; f, 14/35 standard taper joint; g, inlet rubber tubing during gassing and vacuum line outlet after gassing; h, outlet during gassing and inlet afterwards.

standard solution of reduced cytochrome c to determine the diameter of the cell.

In general, the riboflavin was added in excess of the cytochrome on an equivalent basis, since losses of leucoflavin were expected in view of the imperfections of the technique. In a typical experiment, with the apparatus described above, 0.5 ml. of 2.74×10^{-4} m CyFe⁺⁺⁺ (1.37 $\times 10^{-7}$ mole), 1.0 ml. of 10^{-1} m phosphate buffer, pH 7.3, and 1.35 ml. of H_2O were placed in the bottom part of the tube. The sintered glass funnel contained 0.15 ml. of 6.8×10^{-4} m riboflavin (1.02×10^{-7} mole), 0.5 ml. of H_2O , and about 1 mg. of Pd black. Thus the riboflavin was in 50 per cent excess over the

cytochrome. Readings were begun in the Beckman apparatus within 3 minutes after mixing of leucoflavin and cytochrome. At that time only 1.53×10^{-8} mole of CyFe⁺⁺⁺ was left unreduced. This represents 89 per cent reduction of the added cytochrome. Readings were taken 15 minutes longer, but no additional reduction occurred. From this it can be concluded that the reduction of cytochrome by leucoflavin is a very rapid process. The incompleteness of the reduction was probably the result of some retention of leucoflavin on the sintered glass funnel or imperfectly anaerobic conditions. When 2 to 3 equivalents of leucoflavin were present, reduction proceeded to 96 to 98 per cent and, as far as we could judge, it was instantaneous. In one experiment, in which cytochrome was in good excess over leucoflavin, the reduction of CyFe+++ was rapid at first, but slowed down toward the end of the reaction. In general, it may be stated that the reaction of leucoflavin with ferricytochrome is too fast to permit the accurate measurement of reaction rates. A corollary of this observation is the fact that in the over-all reduction of cytochrome by DPNH₂ the first step (oxidation of DPNH₂ by riboflavin) is probably the rate-limiting reaction.

In an attempt to determine the stoichiometry of the reaction, leuco-flavin $+ 2\text{CyFe}^{+++} \rightarrow \text{riboflavin} + 2\text{CyFe}^{++} + 2\text{H}^+$, we modified the technique slightly by washing down the leucoflavin from the sintered glass funnel with several portions of O_2 -free water. The tube containing all components was then allowed to react for 15 minutes, at which time it was opened and made to 25 ml. in a volumetric flask. An aliquot was then read in conventional Corex cuvettes. The results are summarized below.

Riboflav	in added initially	2.72	×	10 ^{−7} mole
CyFe++-	added initially	8.54	×	10-7 "
"	found at end of reaction	3.99	×	10-7 "
"	reduced	4.55	X	10 ⁻⁷ mole
	$Yield = \frac{4.55 \times 10^{-7}}{2 \times 2.72 \times 10^{-7}} \times 100 = 84\%$			
	$\frac{1}{2 \times 2.72 \times 10^{-7}} \times 100 = 84\%$			

In other experiments the yield varied from 75 to 89 per cent. In view of the possible losses of leucoflavin inherent in the imperfections of the technique, this was considered a satisfactory substantiation of the reaction postulated in Equation 2.

 $DPNH_2$ -Riboflavin-O₂ system—In the study of the first part of the overall reaction (Equation 1) advantage was taken of the rapid oxidation of leucoflavin by atmospheric O₂. In this system, then, riboflavin mediated electron transfer from DPNH₂ to O₂; the latter thus replaced ferricytochrome c as the oxidizing agent. Samples of DPNH₂, produced by enzymatic or by hydrosulfite reduction, were allowed to react with riboflavin

under aerobic conditions, and the disappearance of DPNH₂ was measured by the decrease in the intensity of the band at 340 m μ . The reactions were carried out in test-tubes kept in a thermostat at the desired temperature, and aliquots were momentarily transferred into quartz cuvettes when readings were taken. Since riboflavin absorbs significantly in the ultraviolet, experimental vessels were read against a blank containing all constituents, including riboflavin, except the reduced nucleotide. Preliminary measurements indicated that leucoflavin was reoxidized by the dissolved O₂ as rapidly as it was formed; therefore oxygenation made no difference in the rate of the reaction. When riboflavin was left out of the reaction mixtures, the 340 m μ band did not change for at least 30 minutes,

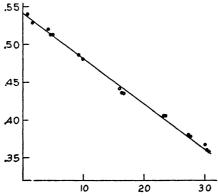


Fig. 5. Aerobic oxidation of enzymatically reduced DPN as catalyzed by riboflavin. Abscissa, time in minutes; ordinate, $1 + \log D_{340}$. The slope of the line k = 6.0×10^{-3} . The reaction mixture contained 3.0 ml. of 10^{-1} m phosphate, pH 7.3, 0.6 ml. of 2×10^{-3} m riboflavin, 0.15 ml. of H₂O, and 1.5 ml. of enzymatically produced DPNH₂ (see the text). Temperature, 30°; pH 7.38. Readings taken in 1 cm. quartz cuvettes against a corresponding blank without DPNH₂.

indicating that the decrease in the intensity of this band was a reliable measure of oxidation of DPNH₂.

The results of a very extensive series of experiments on the oxidation of DPNH₂ and TPNH₂ by riboflavin may be summarized as follows: The kinetics of the reaction are strictly first order. The velocity constant is proportional to the riboflavin concentration, the latter behaving as a true catalyst.

Fig. 5 represents a typical experiment on the oxidation of enzymatically reduced DPNH₂ by O₂, as catalyzed by riboflavin. A large batch of DPNH₂ was prepared by incubating ethanol, DPN, and alcohol oxidase in the presence of 0.1 per cent glycine and 1.4×10^{-2} m pyrophosphate, with 5×10^{-2} m dimedon at 32°. When the reduction was complete as

indicated by readings at 340 m μ , p-chloromercuribenzoate was added to give a final concentration of 2×10^{-4} m. This stopped the action of the enzyme without interfering with subsequent use of the nucleotide preparation (18). The corresponding blank was similarly treated except that no DPN was present in the incubation mixture. Under these conditions 0.218 μ m of DPNH₂ per ml. was formed.

An aliquot of this DPNH₂ solution and an aliquot of the blank were added at zero time to riboflavin in phosphate buffer at 30°, and readings were started. The results are given in Fig. 5, in which the abscissa represents the elapsed time in minutes; the ordinate is $1 + \log D_{340}$. The slope of the resulting straight line, $k = 6.0 \times 10^{-3}$, is a measure of the velocity of the reaction and depends solely on the concentration of the catalyst. It can also be seen that the rate of oxidation of DPNH₂ is a relatively slow process, compared to the very rapid reoxidation of leucoflavin by ferricy-tochrome described in the previous section. Thus in the over-all reaction the reduction of riboflavin is probably the rate-limiting step. Similar first order curves were obtained by the use of hydrosulfite-reduced DPN, as well as with TPNH₂ and NMNH₃.

The catalytic function of riboflavin in this reaction is demonstrated in Fig. 6, in which the velocity constant, k, is plotted against the concentration of riboflavin. Thus riboflavin behaves in the same manner as cytochrome reductase in the reductase assay (3), inasmuch as the reaction rate constant is strictly proportional to the concentration of the flavin.

Reduction of Cytochrome under Anaerobic and Aerobic Conditions—In the experiments hitherto discussed the reduction of cytochrome was measured under aerobic conditions. In view of the known tendency of reduced flavins to react with O₂, it would be expected that a satisfactory stoichiometric relationship between DPNH₂ oxidized and CyFe⁺⁺⁺ reduced could be obtained only in the absence of O₂. The measurement of this relationship was one of the primary purposes of the succeeding experiment. Another purpose was to verify the prediction that the rate of the over-all reaction is limited by the first step; *i.e.*, the oxidation of DPNH₂ by riboflavin.

The enzymatically reduced DPN solution used in these experiments was that used in the experiment recorded in Fig. 5, which was carried out at the same time.

An aliquot of the DPNH₂ solution was placed in the sintered glass funnel of our anaerobic vessel (Fig. 4). All other constituents of the solution, including cytochrome, were in the main tube. A parallel experiment

⁶ We have defined k, the velocity constant expressed in arbitrary terms, as $-d(1 + \log D)/dt$, where t is the time in minutes and D is the optical density at 340 m μ measured in 1 cm. cells. Also $k = -d \log \text{DPNH}_2/dt$.

was set up under aerobic conditions. The pH, temperature, and concentration of the riboflavin were identical with those in the experiment on the aerobic oxidation by riboflavin and O₂ (Fig. 5). The results of these aerobic and anaerobic reactions with cytochrome are represented in Fig. 7.

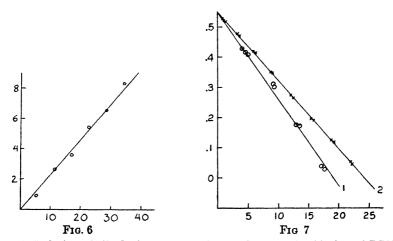


Fig. 6. Relation of riboflavin concentration to the rate of oxidation of DPNH₂. Abscissa, moles per ml. of riboflavin \times 10⁻⁸; ordinate, $-d(1 + \log D)/dt \times 10^3$. Each point represents the average of at least two experiments. The reaction mixture contained 1.5 ml. of 10⁻¹ m phosphate, pH 7.4 to 7.5, 0.75 ml. of riboflavin solution, 2.1 ml. of H₂O, and 0.9 ml. of 5.33 \times 10⁻⁴ m DPNH₂, reduced by hydrosulfite immediately before the experiment. Temperature, 30°. Light absorption read in quartz cells at 340 m μ .

Fig. 7. Anaerobic and aerobic reduction of CyFe⁺⁺⁺ in the presence of riboflavin. Abscissa, time in minutes; ordinate, (log CyFe⁺⁺⁺) + 8. Curve 1, in H₂; Curve 2, in air. In the anaerobic experiment the tube contained 1.9 ml. of 10^{-1} m phosphate, pH 7.3, 0.4 ml. of 2×10^{-3} m riboflavin, 0.2 ml. $\approx 1.26 \times 10^{-7}$ mole of cytochrome c, and 1.0 ml. of DPNH₂ as in Fig. 6. The experiment was performed in the reaction vessel shown in Fig. 5, with the DPNH₂ held on the sintered glass funnel during the 30 minutes gassing with H₂. The reaction started upon mixing of the DPNH₂ with the other constituents. The aerobic tube contained 2.85 ml. of 10^{-1} m phosphate, pH 7.3, 0.6 ml. of 2×10^{-3} m riboflavin, 0.3 ml. $\approx 1.85 \times 10^{-7}$ mole of cytochrome c, and 1.5 ml. of the same DPNH₂. Temperature, 30° ; pH 7.4 in both experiments. Measurements at 550 m μ .

Both the aerobic and the anaerobic reduction of cytochrome c, like the aerobic oxidation of DPNH₂, followed a first order reaction.

The stoichiometry of the over-all reaction can be calculated as follows: By taking the antilogs in the ordinate of Fig. 7, the (CyFe⁺⁺⁺) at zero time was 3.626×10^{-8} mole per ml. At 15 minutes (CyFe⁺⁺⁺) = 1.274×10^{-8} mole per ml. Thus, under anaerobic conditions, 2.35×10^{-8} mole per ml. of CyFe⁺⁺⁺ was reduced.

From Fig. 5, if we take the antilog corresponding to t=0 and t=15 minutes, the corresponding D values are 0.348 and 0.283, respectively. The difference $(\Delta D)=0.065$ for the 15 minute period. From the molar extinction coefficient (10), (DPNH₂) in moles per ml. = $D_{440}/(5.57\times 10^6)$. Hence $0.065/5.57\times 10^{-6}=1.17\times 10^{-8}$ mole per ml. of DPNH₂ oxidized. Since 1 mole of DPNH₂ ≈ 2 moles of CyFe⁺⁺⁺, the corresponding equivalent in terms of cytochrome is 2.34×10^{-8} mole per ml. This is in excellent agreement with the figure obtained from anaerobic measurements of CyFe⁺⁺⁺ reduction at 550 m μ . This finding substantiates the fact that under anaerobic conditions in the over-all process the first step, *i.e.* the reduction of riboflavin, is the rate-limiting one, and further proves the suggested stoichiometrical relationships.

Inspection of Fig. 7 also reveals that aerobically the rate of reduction of cytochrome is only about two-thirds of the anaerobic rate.⁷ The difference represents the extent of competition by dissolved oxygen; *i.e.*,

TABLE II
Relative Rates of Oxidation of DPNH2, TPNH2, and NMNH2

Substrate	Rate of oxidation
DPNH ₂ TPNH ₂ NMNH ₂	97

Rates calculated from first order velocity constant; measurements at 340 m μ . pH 7.3; temperature, 38°. Riboflavin, 22.8 \times 10⁻⁸ mole per ml.

the amount of leucoflavin removed via O_2 . This is not a fixed ratio, of course, and the competition by O_2 will depend on the relative amounts of $CyFe^{+++}$ and O_2 present.

Oxidation of Various Nucleotides by Riboflavin—In view of the relative simplicity of the ultraviolet measurements as compared with the complexity of the anaerobic technique necessary for the quantitative reduction of cytochrome, spectrophotometry at 340 m_{\mu} was used for comparison of the rates of oxidation of DPNH₂, TPNH₂, and NMNH₂. The rate of oxidation of DPNH₂, measured simultaneously with the other two nucleotides, was taken as 100 in Table II. The 78 per cent pure dried preparation of DPNH₂ and the NMNH₂ were those described under "Materials and methods." TPNH₂ was the product of enzymatic reduction by the Zwischenferment system. The latter was inactivated by maintaining it at pH 13 for 6 minutes at 25°; the resulting solution was then neutralized. This treatment left the reduced TPN unaffected.

⁷ The $-d \log \text{CyFe}^{+++}/dt$ values are 0.0302 and 0.0227, respectively.

In two experiments reduced nicotinamide mononucleotide was oxidized 34 to 38 per cent faster than DPNH₂, whereas in a single experiment TPNH₂ was oxidized at very nearly the same rate as DPNH₂ (Table II). All three compounds were oxidized according to a first order reaction.

Relative Efficiency of Various Flavins As Catalysts for Oxidation of DPNH₂—With the technique of ultraviolet spectrophotometry to measure oxidation of reduced nucleotides, the relative efficiency of various flavin derivatives as catalysts of the reaction was determined. Two minor modifications were made in the experimental method. Since only small amounts of FM and FAD were available, the concentration of flavin was lower than in previous experiments. A riboflavin control, however, at the same concentration, was run with each flavin. Secondly, when isoriboflavin was used, the time for 50 per cent disappearance of the substrate (DPNH₂) was

Table III

Comparison of Catalytic Efficiency of Various Flavins

Catalyst	Relative rate of oxidation
Isoriboflavin	257
Riboflavın	100
" phosphate	59
Flavin-adenine dinucleotide	34
Alloxazine	0

Isomboflavin, riboflavin, and alloxazine compared at 5.7 \times 10⁻⁸ and at 11.4 \times 10⁻⁸ mole per ml; other flavins compared with riboflavin at 6.36 \times 10⁻⁸ mole per ml. Source of DPNH₂, Ohlmeyer preparation (11); buffer, 5.7 \times 10⁻² M phosphate, pH 7.35, temperature, 30°, measurement at 340 m μ

calculated, since this reaction followed no definite order. It was found that the reciprocal time for 50 per cent reduction was strictly proportional to the isoriboflavin concentration; this was also true with all other flavins tested. In Table III, the catalytic activity of riboflavin is taken as 100; the rates for the other flavins are calculated from the first order velocity constant, except for isoriboflavin, as noted above.

Alloxazine had no catalytic activity in the reaction. This would be expected from its relative stability to reducing agents. Among the natural compounds, the activity was greatest with riboflavin, but the analogue, isoriboflavin, had by far the greatest activity. It will be recalled that in the aerobic cytochrome reduction test, with TPNH₂ as substrate, isoriboflavin was also over twice as active as riboflavin. Under anaerobic conditions, with DPNH₂ as substrate, the rate of reduction of ferricytochrome c by isoriboflavin followed exactly the same type of curve as was obtained in the ultraviolet measurements, showing once more that the

oxidation of the reduced pyridine nucleotide limits the rate of the over-all reaction. The relative ratio of activities of isoriboflavin and riboflavin in the anaerobic cytochrome test was 2:1, in fair agreement with the findings in Table III.

Kinetics—From the standpoint of kinetics, the simplest experimental conditions exist when the disappearance of the 340 m μ band is measured; viz., the oxidation of reduced pyridine nucleotides by riboflavin and O₂. Since riboflavin acts as a true catalyst in this reaction, only the concentration of the pyridine nucleotides changes. Accordingly, the change in concentration of the latter should determine the reaction order, and, in fact, this reaction regularly yielded kinetics of the first order, regardless of the initial concentration of the pyridine nucleotides used as substrates for the oxidation. (Isoriboflavin catalysis of the reaction is an exception to this statement.) The velocity constant, however, is proportional to the flavin concentration, as expected in a catalytic reaction.

Since measurements of the rate of oxidation of leucoflavin by ferricytochrome c indicated that this step was very rapid, it was to be expected that the rate of the first step should determine the rate of the over-all reaction under *anaerobic* conditions. As noted above, this was the case; the reduction of cytochrome c measured anaerobically followed the same first order reaction as the first step.

The third and most complex case is the over-all reaction under *aerobic* conditions, for then a competition between dissolved O₂ and CyFe⁺⁺⁺ for the leucoflavin occurs and results in a decreased yield of ferrocytochrome. The reaction is too rapid to be compatible with a trimolecular collision and it must therefore be assumed that on reacting with 1 molecule of CyFe⁺⁺⁺ the leucoflavin is oxidized to the semiquinone state. This free radical could then be oxidized either by a 2nd molecule of CyFe⁺⁺⁺ or by O₂.

A high order of regularity for a reaction with so many variable factors could not be expected; all that could reasonably be predicted was that the rate should always be less than that under anaerobic conditions, and this was invariably true. The reaction order varied from a strict first order (as in Fig. 7) to second order (as in Table I and Fig. 1); in other instances it was not amenable to simple mathematical treatment. In the numerous instances in which a straight line was obtained when the reciprocal of the ferricytochrome concentration was plotted against time, it might possibly be inferred that the majority of the leucoflavin reacted with O₂, and the reduction of CyFe⁺⁺⁺ became the limiting factor, reacting with the dihydroflavin in the proportion 2:1.

Note on Stability of DPNH₂—Rather early in this investigation many attempts were made to study the influence of pH on the reaction by ultra-

violet spectrophotometry in the DPNH₂-riboflavin-O₂ system. While it may be generally stated that between pH 9 and 5.5 the rate increases as the pH decreases, strictly quantitative measurements were hampered by the well known instability of dihydronicotinamide compounds to acidity. At and below pH 6 there was a slight but significant decrease in the D_{240} value of DPNH₂ controls in the course of a standard experiment.

During these studies an as yet unexplained observation was made concerning the behavior of DPNH2 solutions. Using the standard conditions for measurement of DPNH2 oxidation at 340 mµ, as in Fig. 5, but substituting a hydrosulfite-reduced DPNH2 as substrate, we measured the velocity constant at a given pH and temperature. Several hours or a day later another aliquot was taken from the same DPNH2 solution and, though the absorption of the latter at 340 m_{\mu} was unchanged, the velocity constant of its oxidation by riboflavin was in many instances increased 20 to 100 per cent. In two instances, after 2 to 3 days storage in the frozen state at pH 7.6 to 7.8, DPNH2 solutions yielded a velocity constant 5 times as high as had been obtained with fresh solutions. It was first thought that the gradual formation of colloidal sulfur on standing might be responsible, but dry preparations of DPNH2 (11), freed from inorganic salts, behaved qualitatively in the same way. Since fresh solutions of riboflavin and those several days old gave the same k value on the same DPNH₂, we felt that some transformation occurred in the nucleotide on standing which was not reflected by the D_{340} value. It might be added that TPNH₂, DPNH₂, NMNH₂, and even dihydronicotinamide riboside have approximately the same coefficient of extinction at 340 mµ; thus considerable variation in the molecule may be brought about which cannot be detected by spectrophotometric means. It must be emphasized that this high rate was not regularly observed with aged solutions of DPNH2. In spite of much effort expended to determine the cause of this phenomenon, we are still at a loss to explain it. It can be avoided, however, by reducing the DPN immediately before use or by storing the nucleotide in the dry state (11) and using a solution of the dried material immediately after dissolving it. When the solution of the nucleotide had to be preserved for even a few hours, it was frozen by means of dry ice. In addition the true DPNH2 content of preparations was ascertained by means of alcohol oxidase. Under these conditions reliable values were obtained.

DISCUSSION

On the basis of the evidence presented, it may be concluded that riboflavin, its nucleotides, and analogues are capable of catalyzing the reaction between dihydropyridine nucleotides and ferricytochrome c. The reason why flavoproteins are eminently suited for this catalytic rôle has been pointed out by Ball (2): they act as mediators between a 2 electron donor (pyridine nucleotides) and a 1 electron acceptor system (cytochrome c), with the intermediate formation of a relatively stable free radical of the semiquinone type (19). There is every reason to believe that free flavins act in the same manner. The reaction is thermodynamically feasible in view of the oxidation-reduction potentials: $E'_0 = +0.262$ volt for cytochrome c at neutral pH (20); approximately -0.250 volt at pH 7.8 and 30° for flavin dinucleotide and -0.232 volt for riboflavin (21); $E'_0 =$ about -0.260 volt for pyridine nucleotides (22). Although the potential of free flavin nucleotides and of riboflavin is 120 mv. or more negative than that of the corresponding flavoproteins, and hence lies close to the range of DPN, in the presence of an oxidizing agent like O_2 or CyFe⁺⁺⁺ free flavins are well suited to be mediators of the oxidation.

Since ferricytochrome c is easily reduced by a number of agents, such as —SH compounds, $H_2 + Pt$, hydrazine compounds, etc., its reduction by leucoflavins is not surprising. A more striking feature of these observations is the demonstration that free flavins are able to oxidize dihydropyridine nucleotides with comparative ease. The relatively small potential span in this step might explain why this reaction should limit the rate of the over-all process.

The demonstration that free flavins can fulfil the rôle of cytochrome reductases in no way implies that this non-enzymatic process has a significant biological rôle. Although direct comparison of the turnover number of flavins with that of the flavin mononucleotide in cytochrome c reductase (TPN) is quite difficult, careful examination of the data presented reveals that the enzymatic process is far more efficient in terms of the flavin concentration required to reduce a given amount of cytochrome c per unit of time. The reasons why attachment of the flavin to the apoenzyme enhances its turnover number may now be more clearly stated. First, in such a holoenzyme combination the stability of the semiguinone radical might be increased, thus enhancing the chance of reaction with a 2nd molecule of ferricytochrome. Second, a relatively tight complex formation between reductase and cytochrome c (3) at the specific surface of the protein increases the probability of reaction with cytochrome and decreases loss of leucoflavin by autoxidation, an inefficient aspect of the non-enzymatic reaction. Third, formation of a relatively tight complex between the flavoenzyme and the reduced pyridine nucleotide (3) as compared with a chance collision would increase the rate of the limiting reaction; i.e., reduction of the flavin by the dihydropyridine group. The same would be accomplished by raising the potential of the flavin by attachment to a specific protein (2) and thereby increasing the potential span between DPNH₂ and flavin, if there is indeed a direct relation between potential

difference and reaction rates. Lastly, the specificity of the mediator is increased by combination with a protein. While riboflavin phosphate reacts as readily with DPNH₂ as with TPNH₂ or even NMNH₂, in combination with the apoenzyme from yeast it is specific for TPNH₂.

Naturally, the protein component required to fulfil these needs has to be a highly specific one; thus, addition of crystalline serum albumin at a concentration of 1 mg. per ml. did not significantly increase the reaction rate in our system.

The reason for the differences in the catalytic efficiencies of various flavins in our system are not clear. They do not seem to be related to the E'_0 value, inasmuch as flavin nucleotides, with a more negative potential than riboflavin, were less efficient, but isoriboflavin, with the same potential as riboflavin (23), was over twice as active.

It is hoped that these experiments provide a sound chemical basis for the function of the isoalloxazine nucleus as a mediator of electron transfer between dihydropyridine nucleotides and cytochrome c or molecular O₂, upon which function the most important biological rôle of riboflavin is built.

SUMMARY

- 1. The non-enzymatic reduction of ferricytochrome c by reduced pyridine nucleotides as catalyzed by various flavins has been demonstrated.
- 2. Of the pyridine nucleotides studied, DPNH₂ and TPNH₂ were oxidized by flavins at about the same rate; NMNH₂ at a faster rate. Of the flavins studied, the most effective catalyst was isoriboflavin, riboflavin, FM, and FAD being relatively less efficient in the order named. Alloxazine was unable to catalyze this reaction.
- 3. The reduction of cytochrome was shown to proceed in two steps; i.e.,

$$DPNH_2 + riboflavin \rightarrow DPN + leucoflavin$$

Leucoflavin + $2CyFe^{+++} \rightarrow riboflavin + 2CyFe^{++} + 2H^+$

The first reaction proceeded as a first order reaction aerobically; the rate constant was proportional to the amount of flavin used as catalyst. The second reaction was almost instantaneous. The over-all reaction, carried out anaerobically, was limited by the rate of the first step and proceeded as a first order reaction. By determination of the amount of DPNH₂ oxidized in the first step and simultaneous measurement of the CyFe⁺⁺⁺ reduced in the anaerobic over-all reaction, it was shown that 2 CyFe⁺⁺⁺ molecules are reduced for 1 DPNH₂ oxidized. Aerobically, the over-all reaction was slower and no longer followed a first order reaction, owing probably to a competition between O₂ and CyFe⁺⁺⁺ for leucoflavin.

4. The probable rôle of the protein moiety of cytochrome reductases in enhancing the specificity and turnover number of the flavins is discussed on the basis of these findings.

BIBLIOGRAPHY

- Warburg, O., and Christian, W., Naturwissenschaften, 20, 688, 980 (1932); Biochem.
 Z., 254, 438 (1932).
- 2. Ball, E., Cold Spring Harbor Symposia Quant. Biol., 7, 100 (1939).
- 3. Haas, E., Horecker, B. L., and Hogness, T. R., J. Biol. Chem., 136, 746 (1940).
- 4. Warburg, O., and Christian, W., Biochem. Z., 298, 150 (1938).
- 5. Kuhn, R., Rudy, H., and Weygand, F., Ber. chem. Ges., 69, 1543 (1936).
- 6. Keilin, D., and Hartree, E. F., Biochem. J., 39, 289 (1945).
- Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 188 (1945).
- 8. Theorell, H., Biochem. Z., 285, 207 (1936).
- 9. Haas, E., J. Biol. Chem., 155, 333 (1944).
- 10. Gutcho, S., and Stewart, E. D., Anal. Chem., 20, 1185 (1948).
- 11. Ohlmeyer, P., Biochem. Z., 297, 66 (1938).
- 12. Kornberg, A., J. Biol. Chem., 174, 1051 (1948).
- 13. Meyerhof, O., and Junowicz-Kocholaty, R., J. Biol. Chem., 149, 71 (1943).
- 14. Cori, G. T., Slein, M. W., and Cori, C. F., J. Biol. Chem., 173, 605 (1948).
- 15. Negelein, E., and Wulff, H. J., Biochem. Z., 293, 351 (1937).
- 16. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 125, 171 (1938).
- 17. Theorell, H., Biochem. Z., 279, 463 (1935).
- 18. Barron, E. S. G., and Singer, T. P., J. Biol. Chem., 157, 221 (1945).
- 19. Michaelis, L., and Schwarzenbach, G., J. Biol. Chem., 123, 527 (1938).
- 20. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., J. Biol. Chem., 124, 11 (1938).
- 21. Michaelis, L., Schubert, M. P., and Smythe, C. V., J. Biol. Chem., 116, 587 (1936).
- 22. Ball, E. G., and Ramsdell, P. A., J. Biol. Chem., 131, 767 (1939).
- Tishler, M., Pfister, K., Babson, R. D., Ladenburg, K., and Fleming, A. J., J. Am. Chem. Soc., 69, 1487 (1947).

THE RELATIVE RATES OF METABOLISM OF NEUTRAL FAT AND PHOSPHOLIPIDES IN VARIOUS TISSUES OF THE RAT*

BY ALEXANDER PIHL AND KONRAD BLOCH

(From the Department of Biochemistry and the Institute of Radiobiology and Biophysics, University of Chicago, Chicago)

(Received for publication, October 17, 1949)

Isotopic tracers have been employed to investigate numerous aspects of lipide synthesis by animal tissues, both in vivo and in vitro. studies attention has been centered on the formation of the carbon chains of the fatty acids, but little information has become available on the rate of entry of fatty acids into the ester linkages of various lipides. Ehrlich and Waelsch (1) compared the isotope concentration in the fatty acids of neutral fat and phosphatides in rat muscle after the administration of heavy water and found a small difference in the deuterium concentration of the two fractions. In the experiments of Barrett, Best, and Ridout (2), the feeding of deuterated linseed oil led to a somewhat higher deuterium concentration in the phospholipides than in the neutral fat of the liver. These studies, as well as those of Cavanagh and Raper (3), were designed to investigate the transport of fat in the animal body. Valuable information on the rate of synthesis and breakdown of the phospholipides in various animal tissues has been obtained with the aid of P⁸² (4, 5). However, this approach does not permit an evaluation of the relative rates of metabolism of phospholipides and other fatty acid esters.

In view of the belief that phospholipides are particularly active in fat metabolism, it seemed of interest to compare the rates at which fatty acids enter into ester linkages of different lipides. Acetic acid has been shown to serve as a precursor of the higher fatty acids both in vivo (6) and in vitro (7) and isotopic acetate can therefore be used to label the carbon chains of these tissue constituents. In the present investigation the incorporation of isotope from C¹⁴-acetate into fatty acids was studied in liver slices and in the intact rat. The isotope concentrations which were found in the fatty acids of phospholipides and neutral fat were compared and taken as a measure of the turnover of the two lipide fractions.

^{*} Supported by a grant in aid from the Life Insurance Medical Research Fund and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[†] Holder of a Fellowship from Forsvarets Forsknings Institutt, Oslo, Norway Present address, Institutt for Ernaeringsforsknings, University of Oslo, Norway.

EXPERIMENTAL

Isotopic acetic acid was prepared from CH₃MgBr and C¹⁴O₂.

Liver Slice Experiments—Slices were cut by hand from livers of male rats of the Sprague-Dawley strain, weighing 150 to 200 gm. 1.5 gm. aliquots from a large amount of pooled liver slices were placed in 13 ml. of Krebs' bicarbonate buffer. 5 mg. of glycerol, 11 mg. of pyruvic acid, and 5 mg. of C¹⁴-sodium acetate were added to the incubation medium in each flask. The C¹⁴-acetate had an activity of 75,000 c.p.m. The pH was 7.4, and the tissue was incubated for 3 hours in an atmosphere of 95 per cent O₂ and 5 per cent CO₂ at 37°. After incubation, the lipides were isolated as described below.

Feeding Experiments—All rats used in the feeding experiments were white males of the Sprague-Dawley strain, weighing 290 to 300 gm. They were kept separately in metabolism cages and given the lipide-free diet (8) for 3 days before the administration of C¹⁴-acetate was begun.

6 Hour Experiment—To each of four rats was given a single dose of 0.36 mm of C¹⁴-acetate by stomach tube. The acetate had a specific activity of 760,000 c.p.m. 6 hours later the rats were killed by exsanguination under ether anesthesia and the blood was collected. From one of these rats the lipides of the liver, of the pooled internal organs (kidneys, gastro-intestinal tract, testes, lung, heart, and spleen), and of the carcass (tissues remaining after removal of the head) were isolated separately. The tissues of the remaining three rats were combined and the lipides were isolated from the following organs: liver, kidney plus adrenals, the intestinal tract and mesentery.

1 and 3 Day Experiment—Two rats were kept on the lipide-free diet and received 1 mm of C¹⁴-acetate per 100 gm. of rat weight per day in their food. The radioactive acetate had a specific activity of 19,000 c.p.m. After 24 and 72 hours, respectively, the rats were killed and the lipides isolated from liver, the pooled internal organs, and the carcass.

Isolation of Lipides—For isolation of the lipides the tissues were extracted several times with 1:1 ether-ethanol at room temperature. The combined extracts were concentrated in vacuo and the residue was taken up in ether. The ether solution was clarified by centrifugation and the phospholipides were precipitated by the addition of 4 volumes of acetone and of a small amount of MgCl₂ in ethanol (9). After standing in the cold for 24 hours, the precipitate was separated by centrifugation. The supernatant was concentrated and the acetone precipitation was repeated to insure complete removal of the phospholipides. The combined phospholipides were washed several times with ice-cold acetone and reprecipitated from an ether solution with acetone. The material thus obtained is designated as the phospholipide fraction, while all lipide material not precipitable by acetone from an ether solution is referred to as the neutral fat.

Blood—20 ml. of plasma were obtained from the pooled oxalated blood of the four animals in the 6 hour experiment. The plasma was mixed with 120 ml. of 1:1 ether-ethanol, the precipitate was washed with ether, and the filtrate was brought to a small volume in vacuo. The residue was taken up in ether and phospholipides and neutral fat were separated by the procedure described above.

Isotope analyses were carried out in all but one case on the fatty acids obtained by hydrolysis of the two lipide fractions. The lipides were digested by refluxing for 2 hours in 50 per cent ethanol containing 10 per cent KOH in a stream of nitrogen. The alkaline hydrolysate was cooled and extracted several times with petroleum ether to remove unsaponifiable material. The alkaline solution was acidified, the fatty acids were extracted by petroleum ether, and the combined extracts were washed once with 5 per cent acetic acid and several times with distilled H₂O. The solvent was evaporated and isotope analyses were carried out, either on the residual total fatty acids or in some cases individually on the saturated and unsaturated fatty acids which had been separated by the lead salt procedure. Since the amount of phospholipide obtained from the plasma was very small, the material was burned directly for isotope analysis. The isotope concentration of the fatty acids of the plasma phospholipides was calculated by assuming that the plasma phospholipide consisted of lecithin (10, 11).

The fatty acids of the neutral fat fraction are derived from all lipides which are not precipitable by acetone. They include therefore the fatty acids of the triglycerides, of the cholesterol esters, and also the free fatty acids. The amount of free fatty acids present in fresh tissues has been reported to be very small (12). Therefore in the feeding experiments the fatty acids obtained from the various neutral fat fractions were presumably derived from the triglycerides and the cholesterol esters only. On the other hand it has been reported that appreciable amounts of free fatty acids are liberated from phospholipides on autolysis of liver tissue (12). It is therefore likely that in the liver slice experiments the reaction mixture contained some free fatty acids and that these were isolated together with the neutral fat fraction. Since the fatty acids from the phospholipides had a much lower isotope concentration than those from the neutral fat fraction, it is likely that the actual differences in the isotope concentration of the two fractions were greater than the analyses indicate.

From the content of cholesterol esters in various tissues it can be estimated that fatty acids linked to cholesterol may comprise as much as 25 per cent of the fatty acids of the neutral fat. While it is likely that the fatty acids of neutral fat in various organs are mainly derived from triglycerides, cholesterol esters and free fatty acids constitute a relatively large portion of the neutral fat fraction (13) in plasma, in which, there-

fore, the isotope concentration of the fatty acids of the triglycerides may be quite different from that of the fatty acid mixture analyzed.

Analysis for Essential Fatty Acids in Rat Liver—Tissue fat contains polyethenoid fatty acids which are of exogenous origin. These fatty acids, which are part of the unsaturated fatty acid fraction, would not be expected to contain any isotope under the present experimental conditions (14). In order to determine the isotope concentration of the monounsaturated fatty acids it is necessary to know the content of polyunsaturated fatty acids in liver fat. From 60 gm. of rat liver the phospholipides and the neutral fat were separated as described above. The contents of arachidonic, linoleic, and linolenic acids were determined according to the spectrophotometric method of Mitchell, Kraybill, and Zscheile (15). The

Table I

Fatty Acid Composition of Phospholipide and Neutral Fat of Normal Rat

Liver

	Per cent total fatty acids							
	Phosph	Neutral fat						
	(A)	(B)	(A)	(B)				
Arachidonic acid	23.2	23.5	11.5	10.8				
Linolenic acid	0.0	0.0	0.16	0.36				
Linoleic "	7.6	7.6	15.9	16.1				
Oleic acid	39.0	39.1	62.8	64.4				
Saturated fatty acids	30.1	30.9	9.6	8.3				
Iodine No.	126.4		124.2					

oleic acid content was determined from the iodine number, and the per cent of saturated fatty acids was calculated by difference¹ (the results obtained in two such experiments are given in Table I).

Isotope Analyses—All compounds were burned in a micro combustion apparatus and the CO₂ was precipitated as BaCO₃. The BaCO₃ was suspended in methanol and deposited on cups of 3.47 sq. cm. area. The samples were counted with a gas flow counter and corrected for thickness. All C¹⁴ concentrations are reported in counts per minute.

DISCUSSION

The lipides of animal tissues are mixtures of several components and cannot be separated readily into well defined fractions. The two lipide

¹ These analyses were kindly performed by Dr. L. R. Dugan and Miss M. Petheram in the Research Laboratories of the American Meat Institute Foundation.

fractions which have been compared in the present study have been distinguished on the basis of their solubility in acetone. This procedure is commonly employed to separate phospholipides from neutral fat, but it should be realized that each of the two fractions is a mixture of several components. Nevertheless, a comparison of the isotope concentrations in the two fractions should give an indication of the relative metabolic activity of the phosphorus-containing lipides and the so called neutral fat.

Liver Lipides—When rat liver slices were incubated with C¹⁴-acetate as a fatty acid precursor, the fatty acids obtained from neutral fat were found to have an isotope concentration about twice as high as that of the fatty acids from the phospholipides (Table II). Since in rat liver slices acetate carbon is incorporated at a much faster rate into the saturated than into

Table II

Incorporation of C¹⁴-Acetate into Fatty Acids of Neutral Fat and Phospholipides
in Rat Liver Slices

		C14 concentration, c.p.m.							
Experiment No.				Unsaturated fatty ac					
No.		Total fatty acids	Saturated fatty acids	Total	Monounsatu- rated*				
	er anna e senere e service a service a service a service a service de service a servic			(A)	(B)				
1	Neutral fat	132	325	41	59				
	Phospholipides	80	127	15	27				
2	Neutral fat	64	99	29	42				
	Phospholipides	28	50	12	22				

For details, see "Experimental" in the text.

the unsaturated fatty acids (16), differences in fatty acid composition could be responsible in part for these results. However, separate analyses demonstrated that both saturated and unsaturated fatty acids of the neutral fat had a much higher isotope concentration than those of the phospholipides. The data for the unsaturated acids reflect the relative rates of incorporation of the monounsaturated fatty acids only because the polyunsaturated acids do not contain any isotope under the present conditions (14). The actual isotope concentrations of the inonounsaturated fatty acids (Column B, Table II) were calculated by subtracting the content of these unlabeled fatty acids.

In order to investigate whether differences in metabolic rates of various lipides could be detected also in the intact animal, C¹⁴-acetic acid was administered to adult rats for various periods of time. 6 hours after a single administration of labeled acetate by stomach tube the isotope concentra-

^{*} Calculated from the data in Table I.

tion in the total, the saturated, and the unsaturated fatty acids of neutral fat of liver was found to be significantly higher than in the corresponding fractions of the liver phospholipides (Table III). Therefore, in vivo as well as in vitro, the newly synthesized fatty acids are more rapidly incorporated into the ester linkages of neutral fat than of liver phospholipides and it would appear that phospholipides cannot be obligatory intermediates in the synthesis of neutral fat.² Zilversmit, Chaikoff, and Entenman (17) have reached the same conclusion with respect to the rôle of phospholipides in the intestinal absorption of fat. The present findings are also in accord with the results obtained by Cavanagh and Raper (3). These investigators fed deuterated linseed oil to rats and determined the deuterium concentration in the phospholipides and neutral fat of various organs after

TABLE III

Isotope Concentration in Fatty Acids of Neutral Fat and Phospholipides of Liver 6

Hours after Feeding of C¹⁴-Labeled Acetate

		C ¹⁴ concentration, c.p.m.						
Rat No.				Unsaturat	ed fatty acids			
		Total fatty acids	ds acids Total Mon	Monounsatu- rated*				
1	Neutral fat	2600	3100	2500	3600			
	Phospholipide	1500	1900	1200	2100			
2, 3, 4	Neutral fat	2500	2600	2500	3600			
Pooled	Phospholipide	1700	2200	1300	2300			

^{*} Calculated from the data in Table I.

successive time intervals. Their finding that between the 10th and 24th hours following the administration of labeled fat the deuterium concentration in the neutral fat of liver decreased much faster than that of the phospholipides indicates that the replacement of the fatty acid was more rapid in neutral fat.

In the experiments lasting 1 and 3 days (Table IV), in which the animals received the labeled acetate admixed with the diet, the isotope concentrations of the fatty acids of the neutral fat and phospholipides of liver were essentially the same. The fact that differences can no longer be observed after these time intervals suggests that the regeneration of the ester bonds is a very fast process in both lipides.

Plasma Lipides—The isotope concentrations of the lipides of plasma in

² This conclusion will not be valid if one of the components of the phospholipide fraction has a very much higher rate of turnover than is indicated by the C¹⁴ concentration in the total mixture. However, no evidence exists at present to support the latter assumption.

the 6 hour experiment were found to be very high and to be exceeded only by those of the lipides in liver (Table V). Here also the isotope concentration of the fatty acids of the neutral fat fraction is higher than that of the phospholipide. Since only total fatty acids were analyzed, it is important to consider the fatty acid composition of the plasma lipides. An exact fatty acid analysis of these lipides is available only for ox plasma (13). In contrast to the neutral fat from various tissues, that of plasma contains only a small percentage of triglycerides and a large amount of free fatty

Table IV

Isotope Concentration in Total Fatty Acids of Neutral Fat and Phospholipides of Rat

Tissues after Feeding of C¹⁴-Acetate

			C ¹⁴ concentration, c.p.m.							
Rat No. Duration		Liver		Pooled internal organs		Carcass				
		Neutral fat	Phospho- lipide	Neutral fat	Phospho- lipide	Neutral fat	Phospho- lipide			
	hrs.									
5	24	230	260	29	115	23	32			
6	72	340	360	42	155	31	76			

Table V

Isotope Concentration in Total Fatty Acids of Neutral Fat and Phospholipides 6

Hours after Feeding of C¹⁴-Labeled Acetate

	C14 concent	ration, c.p.m.	
Pooled internal organs	Neutral fat	Phospholipides	
Pooled internal organs	77	169	
Kidney plus adrenals	68	344	
Intestinal tract	118	430	
Mesenterial fat	29	75	
Carcass	81	87	
Plasma	1600	1400	

acids and cholesterol ester. The fatty acids linked to cholesterol are known to consist to a large extent of essential fatty acids. It is therefore likely that the isotope concentration in the saturated and monounsaturated fatty acids of the neutral esters in plasma is appreciably higher than the analytical data indicate.

In view of the fact that the phospholipides comprise a relatively small fraction of the total plasma lipides (13) and also have a relatively low isotope concentration, it is unlikely that the phospholipides are the major vehicle for fat transport.

The isotope concentration of the fatty acids of the plasma phospholipides

was approximately 90 per cent that of the fatty acids in the liver phospholipides. Since liver is the only organ in which the isotope concentration of the phospholipides is greater, the plasma phospholipides must have originated from this organ. The present data are in agreement with previous experiments with P³² (5, 18, 19), which demonstrated that the liver is the site of synthesis for plasma phospholipides. A direct comparison of the radioactivity in the neutral fat fraction of plasma and liver has little meaning, as their composition is widely different.

Internal Organs—In the 6 hour experiment the relation between the isotope concentrations of phospholipides and neutral fat in the combined internal organs and in those internal organs which were separately analyzed was found to be the reverse of that existing in the liver (Table V). At all time intervals the radioactivity of the fatty acids from phospholipides exceeded that of the fatty acids from neutral fat several fold (Table IV). It is of interest that this is true not only for the parenchymatous lipides of the kidney and adrenals, but also for mesenterial fat, which is a representative depot fat. While it is evident that in these internal organs the rate of turnover of phospholipide is greater than that of neutral fat, the present data do not indicate to what extent the lipides in these organs are replaced by interchange with the lipides of the blood and by synthesis in situ.

The level of radioactivity in the neutral fat from all extrahepatic tissues, including carcass, was about the same. On the other hand, the metabolic activity of the phospholipides differed widely from organ to organ and was lowest in carcass and mesenterial fat. While in the extrahepatic tissues discussed above the isotope concentration in the phospholipides was several times greater than that of the neutral fat, this was not the case for the lipides from carcass. In one of the three experiments (Table IV) the isotope concentration of the phospholipides was significantly higher than in the neutral fat, but the data for carcass as a whole indicate relatively small differences in metabolic activity between the two fractions.

The present findings do not permit a generalized statement concerning the relative metabolic activity of phospholipides and neutral fat in the animal organism. Thus the results obtained for liver indicate that ester bond formation is a faster process for triglycerides than for phospholipides. In this organ the observed radioactivities reflect primarily the synthesis of fat. On the other hand, the appearance of labeled lipides in extrahepatic organs must to a large extent result from the uptake of lipides from the blood stream. Irrespective of the mechanism by which replacement occurs in these organs, these processes are decidedly faster for phospholipides than for neutral fat.

SUMMARY

- 1. The incorporation of isotopic carbon from C¹⁴-acetate into the fatty acids of neutral fat and of phospholipides has been studied in isolated liver and in the intact rat.
- 2. The results obtained show that in liver the rate of regeneration of neutral fat is faster than that of phospholipides. It is concluded that the phospholipides are not obligatory intermediates in the synthesis of triglycerides.
- 3. In all other internal organs, including mesenterial fat, the isotope concentrations found in the phospholipides were markedly higher than in neutral fat.

BIBLIOGRAPHY

- 1. Ehrlich, G., and Waelsch, H., J. Biol. Chem., 163, 195 (1946).
- 2. Barrett, H. M., Best, C. H., and Ridout, J. H., J. Physiol., 93, 267 (1938).
- 3. Cavanagh, B., and Raper, H. S., Biochem. J., 33, 17 (1939).
- 4. Artom, C., Sarzana, G., and Segre, E., Arch. internat. physiol., 47, 245 (1938).
- Hevesy, G., and Hahn, L., Kgl. Danske Videnskab. Selskab, Biol. Medd., 15, 6 (1940).
- 6. Rittenberg, D., and Bloch, K., J. Biol. Chem., 160, 417 (1945).
- 7. Bloch, K., and Kramer, W., J. Biol. Chem., 173, 811 (1948).
- 8. Pihl, A., Bloch, K., and Anker, H. S., J. Biol. Chem., 183, 441 (1950).
- 9. Sinclair, R. G., J. Biol. Chem., 88, 575 (1930).
- 10. Taurog, A., Entenman, C., and Chaikoff, I. L., J. Biol. Chem., 156, 385 (1944).
- 11. Sinclair, R. G., J. Biol. Chem., 174, 343 (1948).
- 12. Fairbairn, D., J. Biol. Chem., 157, 645 (1945).
- 13. Kelsey, F. E., and Longenecker, H. E., J. Biol. Chem., 139, 727 (1941).
- 14. Bernhard, K., and Schoenheimer, R., J. Biol. Chem., 133, 707 (1940).
- Mitchell, J. H., Jr., Kraybill, H. R., and Zscheile, F. P., Ind. and Eng. Chem., Anal. Ed., 15, 1 (1943).
- 16. Bloch, K., Cold Spring Harbor Symposia Quant. Biol., 13, 29 (1948).
- Zilversmit, D. B., Chaikoff, I. L., and Entenman, C., J. Biol. Chem., 172, 637 (1948).
- Hevesy, G., and Aten, A. H. W., Kgl. Danske Videnskab. Selskab, Biol. Medd., 14, 5 (1939).
- 19. Artom, C., J. Biol. Chem., 139, 953 (1941).

THE RATES OF SYNTHESIS OF FATTY ACIDS AND CHO-LESTEROL IN THE ADULT RAT STUDIED WITH THE AID OF LABELED ACETIC ACID*

BY ALEXANDER PIHL,† KONRAD BLOCH, AND H. S. ANKER

(From the Department of Biochemistry and the Institute of Radiobiology and Biophysics, University of Chicago, Chicago)

(Received for publication, October 17, 1949)

The rates of synthesis and destruction of organic tissue constituents in the animal organism were first investigated by Schoenheimer and Rittenberg (1, 2). By administering heavy water to mice they were able to measure the rates of regeneration of fatty acids and cholesterol. Several similar studies have been carried out with mice (3) and rats (4, 5), and approximate values for the turnover of the lipides in liver, carcass, and the total animal body have been reported.

The rate of turnover of a body constituent is not necessarily a constant, but may vary with the animal species, age, the composition of the diet, etc. In several of the previous turn-over studies, fat has not been rigidly excluded from the diet, while in others net formation of tissue constituents took place. In the earlier experiments deuterium has been the tracer throughout, on the assumption that the rate of incorporation of deuterium from the heavy water medium is a true measure of the rate of synthesis of the compound in question. Since no such assumption need be made when a carbon isotope is used in the tracer molecule, the rate of turnover of fatty acids and cholesterol has been reinvestigated with the aid of acetic acid labeled by C¹⁴ in the carboxyl group. The present study had the additional object of determining the extent of utilization of acetic acid in the synthesis of fatty acids and cholesterol.

Adult rats were used as the experimental animals. They were maintained at constant body weight on a diet completely devoid of fat and cholesterol. γ -Phenyl- ρ -aminobutyric acid was added to the diet, and its acetyl derivative isolated from the urines.

EXPERIMENTAL

Isotopic potassium acetate labeled in the carboxyl group with C¹⁴ was prepared from CH₂MgBr and C¹⁴O₂.

- * Supported by a grant in aid from the Life Insurance Medical Research Fund and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.
- † Holder of a Fellowship from Forsvarets Forsknings Institutt, Oslo, Norway. Present address, Institutt for Ernaeringsforsknings, University of Oslo, Norway.

Feeding Experiments—Eight male rats of the Sprague-Dawley strain, weighing 290 to 300 gm. each, were kept separately in metabolism cages and given 12 to 15 gm. per day per rat of the following diet: vitamin-free casein 20, sucrose 65, non-nutritive material 10, and standard salt mixture 5.

To each kilo of the above diet, vitamins were added in the following amounts: thiamine 4 mg., riboflavin 10 mg., calcium pantothenate 16 mg., inositol 1 gm., p-aminobenzoic acid 30 mg., choline 1 gm., folic acid 2 mg., biotin 0.1 mg., β -carotene 5.4 mg., calciferol 0.03 mg., 2-Me-1-4-naphthoquinone 2 mg., α -tocopherol 60 mg., and pyridoxine 4 mg.

The requirement for essential fatty acids was met by administering 15 mg. of methyl linoleate per rat per day. Methyl linoleate and the fatsoluble vitamins, dissolved in tripropionate, were added to the ration each day.

Isotopic acetic acid, having a specific activity of approximately 1 μ c. per mm, was thoroughly mixed with the ration and fed at a level of 1 mm per 100 gm. of rat tissue per day. A sample of the acetic acid was converted to silver acetate. It showed an activity of 19,000 c.p.m., counted as barium carbonate.

Pairs of rats were sacrificed after the 1st, 3rd, 10th, and 30th days (Experiment A). Fatty acids from liver and eviscerated carcass were isolated according to customary methods and separated into saturated and unsaturated fractions by the lead salt procedure. Cholesterol from liver was isolated as the digitonide. Carcass cholesterol was obtained from the unsaponifiable fraction by repeated recrystallizations.

The urines were collected and N-acetylphenylaminobutyric acid was isolated and purified according to the method described previously (6). In the 1, 3, and 10 day experiments the urine excreted by each individual rat during the entire experimental period was pooled for isolation of acetylphenylaminobutyric acid. The isotope concentrations found are, therefore, average values for the respective periods. The quantities of phenylaminobutyric acid added to the diet were 150, 50, 25, and 25 mg. per day, respectively, in the 1, 3, 10, and 30 day experiments. In the 30 day experiment acetylphenylaminobutyric acid was isolated separately from the urine excreted during the first 17 day period and from that excreted during the last 13 days. In another 30 day experiment (Experiment B), two rats received 50 mg. of pr-phenylaminobutyric acid per rat per day. The urines from eight successive periods were collected and analyzed separately. The isotope concentrations of the acetyl groups of acetylphenylaminobutyric acid in Experiment A are given in Table I and those for Experiment B, in Fig. 4.

¹ General Biochemicals, Inc., Chagrin Falls, Ohio.

² Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 15, 311 (1913).

Isotope Analysis—All compounds were burned in a micro combustion apparatus and the CO_2 was precipitated as $BaCO_3$. The $BaCO_3$ was suspended in methanol and deposited on cups of 3.5 sq. cm. area. The samples were counted with a flow gas counter and corrected for thickness. All values are reported in counts per minute. The probable error of the analyses is ± 5 per cent. The C^{14} concentrations in cholesterol samples which had been isolated as the digitonides were obtained by multiplying the analytical values by 3.07. The C^{14} concentrations of the acetyl groups in acetylphenylaminobutyric acid were calculated by multiplying the found values by 6.

Table I

Isotope Concentration of Acetyl Groups of Acetylphenylaminobutyric

Acid: Experiment A

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Ra	t 7	R	at 8
Period of urine collection, days Acetyl groups, c.p.m	1	1	3	3	10	10	1-17	18–30	1-17	18-30
	1540	1460	1650	1550	1900	1 76 0	2000	2050	2150	1900

DISCUSSION

The customary method of calculating the half time³ of a tissue constituent from the rise of its isotope concentration with time is valid only if the isotope concentration of the precursor stays constant throughout the experimental period. It has previously been assumed that after the feeding of labeled acetate the isotope concentration of the acetic acid which is available for endogenous synthesis at any given time is indicated by the isotope concentration of N-acetyl groups which are excreted after the administration of foreign amines (7). The isotope concentration of such acetyl groups does not change with time over feeding periods lasting as long as 3 months, if deuterioacetate is the test substance (8). In the present experiments with acetic acid labeled by a carbon isotope the same procedure has been used. Phenyl-pL-aminobutyric acid was fed simultaneously with the labeled acetate and consecutive samples of the excreted acetyl derivatives were analyzed. As shown in the data of Table I, the isotope concentration of the acetyl groups, contrary to the findings with deuterioacetate, changes with time when carbon-labeled acetic acid is the precursor and increases by approximately 30 per cent in the course of 30 days. It must be assumed that this rise reflects an increase of the isotope concentration of the acetic acid which serves as the precursor for fatty acids and cholesterol. In order to determine the half time it is

³ The time at which the isotope concentration of a body constituent is one-half of the value which would be reached at infinite time.

therefore necessary to recalculate the found isotope values for fatty acids and cholesterol on the basis of the changing isotope concentration of the acetic acid in the metabolic "pool." These values have therefore been adjusted by calculating the isotope concentrations of the tissue constituent in per cent of the average isotope concentration in the acetyl groups excreted during the corresponding time interval.

Fatty Acids—In Fig. 1 are plotted the isotope concentrations in the saturated and unsaturated fatty acids of liver after various time intervals, expressed in per cent of the isotope concentration of the acetyl groups. The saturated fatty acids reach half of their maximal isotope concentrations in less than 1 day, and the unsaturated acids in about 2 days. These

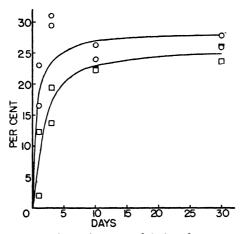


Fig. 1. Isotope concentrations of saturated (\bigcirc) and unsaturated (\square) fatty acids of liver, expressed in per cent of the isotope concentrations of the acetyl groups excreted at corresponding time intervals.

values are in good agreement with the half life time of 1.9 days obtained with D₂O by Stetten and Boxer (5) for the total fatty acids of rat liver.

The estimation of the half time of the liver fatty acids may be subject to considerable error because the isotope concentrations rise very sharply during the initial phase of the experiment. When the half time of a compound is as short as 1 day, variations in food intake, etc., during the experimental period may have an appreciable effect on the isotope concentration of the labeled product. A more accurate value of the half time is therefore not obtainable from feeding experiments with the analytical technique which is used at present.

The isotope concentration of the carcass fatty acids did not reach a maximum within the experimental period of 30 days and consequently the half time could not be determined directly from the data, as was

possible for the liver fatty acids. In the experiments with deuterioacetate of Ponticorvo, Rittenberg, and Bloch (8) the isotope concentration of the carcass fatty acids reached the same final value as the liver fatty acids when the feeding period was extended beyond 1 month. We have therefore assumed that in the present experiment also the isotope concentration in the carcass fatty acids will ultimately attain the same maximal value as the fatty acids of the liver. Furthermore, by plotting the data semilogarithmically with the first order rate equation, I = I = I I, a straight line is obtained only when the values used for I = I I are those of the

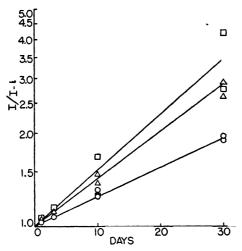


Fig. 2. Isotope concentrations of saturated (\square) and unsaturated (\triangle) fatty acids and cholesterol (\bigcirc) of the carcass, plotted as the function $\ln I/(I-i)=kt$. I and i are expressed in per cent of the isotope concentrations of the acetyl groups excreted at corresponding time intervals.

saturated and unsaturated fatty acids in the liver. This supports the assumption that the isotope concentrations in the fatty acids in carcass will ultimately be the same as in the fatty acids of liver. After plotting of the data according to the above equation (Fig. 2), k can be estimated from the slope of the curve and the half time can be obtained from the relation $t_1 = (\ln 2)/k$. Values of 16 to 17 days for the saturated and 19 to 20 days for the unsaturated fatty acids of carcass are found.

The values obtained in the present investigation are appreciably higher than the half life time of 9 days found by Bernhard and Bullet (4) in experiments on rats with heavy water. This difference is unexpected because the half time should be independent of the nature of the tracer molecule

 $^{^4}$ I, isotope concentrations at infinite time and i, isotope concentration at time t.

used. A variation in the behavior of different rat strains is the only apparent explanation for the differences observed in the two laboratories.

It may be noted that the unsaturated fatty acids are synthesized at a slightly slower rate than the saturated fatty acids while their maximal isotope concentrations are not appreciably lower. As the unsaturated fatty acid fractions include the essential polyethenoid fatty acids which presumably are unlabeled (9), the isotope concentrations of the monounsaturated acids must be at least as high as those of the saturated fatty acids (10). On the other hand, in investigations with heavy water as a tracer, the maximal isotope concentration of the unsaturated fatty acids was found to be not more than half that in the saturated acids from the

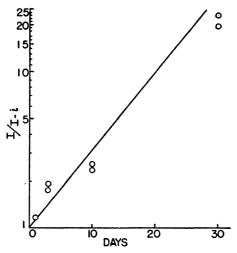


Fig. 3. Isotope concentration of liver cholesterol plotted as the function $\ln I/(I-i) = kt$. I and i were calculated in the same manner as explained in Fig. 2.

same organ (2-4). It should be pointed out that in some of the earlier investigations the diets employed were not rigorously freed of fat. This may account in part for the observed differences. The fact that in the present experiments the saturated and the monounsaturated fatty acids show the same isotope concentration at infinite time suggests that acetic acid is used to the same extent in their synthesis. On the other hand, the differences in the maximal deuterium concentration observed after administration of heavy water probably reflect a difference in the mechanism of synthesis for saturated and unsaturated fatty acids as suggested by Bernhard and Bullet (4) and by findings from this laboratory (11, 12).

Cholesterol—The data for cholesterol have been plotted semilogarithmically as in the case of the fatty acids (Fig. 3). For liver cholesterol a

half time of about 6 days is obtained. Since the isotope concentration in the cholesterol of carcass (Fig. 2) rises slowly and, like that of the carcass fatty acids, does not reach its maximum within the experimental period, the half time has been calculated under the assumption that its isotope concentration reaches the same maximal value as the liver cholesterol. On this basis the half time in carcass is calculated to be 31 to 32 days. Rittenberg and Schoenheimer (2) estimated the half life time of total body cholesterol in mice to be 15 to 25 days.

Utilization of Acetate for Lipide Synthesis—In previous studies (7, 11, 8) the extent of utilization of acetate for the synthesis of a body constituent has been estimated by referring the isotope concentration of the product in question to the isotope concentration of excreted N-acetyl groups. The assumption has been made that the isotope concentration of these acetyl groups is the same as that of the acetate at the site of synthesis.

In the present experiment the maximal isotope concentration in the liver cholesterol amounts to 26 per cent of that in the acetyl groups. If the 2-carbon atoms of acetic acid were utilized equally in cholesterol synthesis, 26 per cent of all carbon atoms would have their origin in acetic acid. Actually in experiments on cholesterol formation in rat liver slices it has been found (13) that the methyl and carboxyl carbons in acetic acid enter cholesterol in a ratio of 1.27:1. It seems likely that in vivo also a larger proportion of the sterol carbon atoms is supplied by the methyl group of acetic acid. Therefore the value of 26 per cent represents the minimal utilization of the acetic acid molecule. This value is substantially lower than previous estimates (7, 14, 8) on acetate utilization for steroid synthesis.

Recently the isotope concentration of some individual carbon atoms of cholesterol, which had been synthesized in liver slices in the presence of carbon-labeled acetate, has been determined (13). The results obtained indicated that every individual carbon atom of cholesterol can be derived from acetic acid. Since no other specific carbon source of cholesterol has been found so far, and since in the present experiment the cholesterol contained only one-fourth of the isotope concentration of the excreted acetyl groups, it would appear that the 2-carbon fragments which serve as the immediate precursor of cholesterol and of the acetyl groups excreted in the urine, respectively, do not have the same isotope concentration. No decision can be made at the present time as to whether there exist other sources of the immediate sterol precursor or whether it is erroneous to assume that the isotope concentration in the excreted acetyl groups reflects the true isotope concentration of acetic acid in the metabolic "pool."

Similar considerations apply to the rôle of acetic acid as a carbon source

for fatty acids. The finding in the present experiments that the maximal isotope concentration in the liver fatty acids was about 25 per cent that of the acetyl groups indicates that at least one-fourth of the carbon atoms of the fatty acids is furnished by acetic acid. The present value of acetate utilization for fat synthesis is in good agreement with previous estimates from experiments on mice with carbon-labeled acetic acid (14) and on rats with deuterioacetic acid (8).

In the case of the fatty acids also, evidence has been obtained that acetic acid can furnish every individual carbon atom of the molecule (14). Again, the values of acetate utilization, calculated from the present and from earlier experiments, can be minimal values only for the reasons mentioned above in the discussion of cholesterol synthesis. The two processes differ, however, in that 2-carbon units derived from pyruvic acid are a source of carbon atoms for the higher fatty acids but do not con-

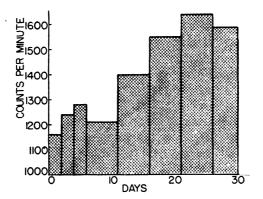


Fig. 4. Experiment B: isotope concentrations of excreted acetyl groups

tribute proportionally to the acetylation of foreign amines (11). The isotope concentration of the N-acetyl groups after feeding labeled acetate is therefore a measure of the acetate pool in a restricted sense only.

The observation that the isotope concentration of the excreted acetyl groups rises gradually on continued feeding of labeled acetate was confirmed by a second long term experiment in which the isotope concentration of the acetyl groups was determined in smaller time intervals. The results are given in Fig. 4. The increase of the isotope concentration above the initial value in the course of the 30 day period was again about 30 per cent. Since the amount of exogenous labeled acetate was constant, the observed rise must be caused by labeled tissue constituents, which in turn are metabolized to yield labeled acetyl groups. Compounds which are known to be synthesized from acetate as a precursor are fatty acids (14), cholesterol (15), heme (7), and purines (16). On the other hand the

formation of acetic acid as a catabolic product has been demonstrated only for fatty acids (6) and for one of the ketogenic amino acids (17). Under the present experimental conditions the higher fatty acids are the only compounds which can contribute significant quantities of labeled acetyl groups to the acetic acid pool. The question arises why the isotope concentration in the excreted acetyl groups increases with time when carbon-labeled acetate is the test substance but stays constant when deuterioacetate is used (8). It is likely that during the breakdown as well as during the synthesis of deuterio fatty acids the major part of the deuterium will be replaced by ordinary hydrogen from the body fluids. The deuterium concentration in a 2-carbon split-product arising during the breakdown of a deuterio fatty acid will thus be too small to affect the isotope concentration of the excreted acetyl groups. On the other hand, such a loss of isotope cannot occur with fatty acids which contain isotopic carbon.

From the data furnished by the present experiment, the quantities of acetyl groups formed by the breakdown of fatty acids can be estimated. In this calculation only the carcass fatty acids will be considered. Under the assumption that a 300 gm. rat has a fatty acid content of 20 gm., with an average half time of 18 days, it is calculated that a total of about 22 mm of acetic acid can be produced from this source per day. This acetate will have the same isotope concentration as the fatty acids from which it is formed. After feeding 3 mm of acetate per rat per day with an isotope concentration of 19,000 c.p.m., the acetyl groups in the 1 day experiment were found to contain about 1500 c.p.m. (Table I). The 3 mm of dietary acetate had therefore been diluted by about 35 mm of unlabeled endogenous acetate. On the 30th day of the experiment, the 3 mm of dietary acetate will mix with the same quantity of endogenous acetate which now, however, has an isotope concentration at least as high as that of the carcass fatty acids; i.e. 360 c.p.m. This contribution will raise the isotope concentration in the mixture of exogenous and endogenous acetate to be employed for the acetylation reaction from 1500 to 1800 c.p.m. The actual isotope concentration in the acetyl groups excreted between the 17th and 30th days was found to have an average value of 2000 c.p.m. Therefore the degradation of the fatty acids in the carcass alone can account for about 60 per cent of the observed rise. The contribution of labeled acetyl groups from fat in other tissues must also be considerable but is difficult to evaluate because the rate of turnover of the fatty acids in the internal organs has not been determined. Moreover, in the case of the liver the observed rate of turnover probably reflects not only the synthesis and breakdown of the fatty acids, but also the transport of fatty acids to and from extrahepatic tissues. In any case the results are consistent with

the assumption that the increasing isotope concentration in the excreted acetyl groups is due to the formation of labeled 2-carbon units from the higher fatty acids. This is in accord with the view that the size of the acetyl pool as indicated by the acetylation reaction can be accounted for by the known metabolic activity of tissue fat (7). Other sources such as carbohydrate may give rise to intermediates which behave like 2-carbon fragments in some respects, e.g. in the synthesis of fatty acids, but which do not contribute proportionally to the acetic acid pool as measured by the acetylation reaction.

SUMMARY

- 1. The rate of synthesis of fatty acids and cholesterol in the adult rat has been determined with the aid of acetic acid labeled by C¹⁴. The half times for the saturated and unsaturated fatty acids were found to be about 1 day and 2 days, respectively, in liver and 16 to 17 days and 19 to 20 days, respectively, in the carcass. The half time of cholesterol was found to be 6 days in liver and 31 to 32 days in carcass.
- 2. Minimal values have been calculated for the utilization of acetic acid in the synthesis of cholesterol and of fatty acids in the adult rat.

BIBLIOGRAPHY

- 1. Schoenheimer, R., and Rittenberg, D., J. Biol. Chem., 114, 381 (1936).
- 2. Rittenberg, D., and Schoenheimer, R., J. Biol. Chem., 121, 235 (1937).
- 3. Bernhard, K., and Schoenheimer, R., J. Biol. Chem., 133, 713 (1940).
- 4. Bernhard, K., and Bullet, F., Helv. chim. acta, 26, 75, 1185 (1943).
- 5. Stetten, D., Jr., and Boxer, G. E., J. Biol. Chem., 155, 231 (1944).
- 6. Bloch, K., and Rittenberg, D., J. Biol. Chem., 155, 243 (1944).
- 7. Bloch, K., and Rittenberg, D., J. Biol. Chem., 159, 45 (1945).
- 8. Ponticorvo, L., Rittenberg, D., and Bloch, K., J. Biol. Chem., 179, 839 (1949).
- 9. Bernhard, K., and Schoenheimer, R., J. Biol. Chem., 133, 707 (1940).
- 10. Pihl, A., and Bloch, K., J. Biol. Chem., 183, 43 (1950).
- 11. Anker, H. S., J. Biol. Chem., 176, 1337 (1948).
- 12. Bloch, K., Cold Spring Harbor Symposia Quant. Biol., 13, 29 (1948).
- 13. Little, H., and Bloch, K., J. Biol. Chem., 183, 33 (1950).
- 14. Rittenberg, D., and Bloch, K., J. Biol. Chem., 160, 417 (1945).
- 15. Bloch, K., and Rittenberg, D., J. Biol. Chem., 145, 625 (1942).
- 16. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., J. Biol. Chem., 166, 395 (1946).
- 17. Bloch, K., J. Biol. Chem., 155, 255 (1944).

THE OCCURRENCE OF γ-AMINOBUTYRIC ACID IN YEAST EXTRACT; ITS ISOLATION AND IDENTIFICATION

By LESTER J. REED

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin)

(Received for publication, August 1, 1949)

Partition chromatography on paper has proved an invaluable tool in separating the amino acids in protein hydrolysates and biological extracts (1–5). The amino acids migrate to characteristic positions on paper chromatograms, as revealed by a color reaction with ninhydrin. During the course of studies with yeast extract (Difco), this material was subjected to mild acid hydrolysis, and the hydrolysate was examined chromatographically. The presence in appreciable quantity of an amino acid of unknown nature was revealed. This amino acid was isolated from the acid-treated yeast extract and identified as γ -aminobutyric acid. Further experiments demonstrated that it is present in the free state in untreated yeast extract.

The isolation was effected by means of partition chromatography on a starch column (6), by use of n-butanol-acetic acid-water as the solvent system. Separation of the amino acids by the column was traced by paper partition chromatography of samples of the effluent.

EXPERIMENTAL

50 gm. of Bacto yeast extract¹ were dissolved in 500 ml. of 6 N hydrochloric acid and the solution was autoclaved for 1 hour at 120°. The cooled digest was treated with Darco G-60 and freed of chloride ions by successive treatments with lead oxide and silver carbonate. The filtrate from these operations was saturated with hydrogen sulfide, decolorized with Darco G-60, and concentrated to a volume of approximately 150 ml. This solution contained 113 mg. of solids per ml.

Partition Chromatography on Paper²—A two-dimensional paper chromatogram of the chloride-free acid digest of yeast extract (Fig. 1) was prepared and developed according to the "ascending" method of Williams and Kirby (7). A comparison of this chromatogram with one obtained by using a mixture of the known naturally occurring amino acids revealed that an amino acid of unknown nature was present in the acid digest of

¹ Obtained from the Difco Laboratories, Detroit, Michigan.

² Whatman No. 1 filter paper was used throughout this work.

yeast extract. In a similar manner the "unknown" amino acid was shown not to be identical with a number of synthetic amino acids.

Several different solvent systems were tested in conjunction with onedimensional paper chromatograms to determine their effectiveness in sep-

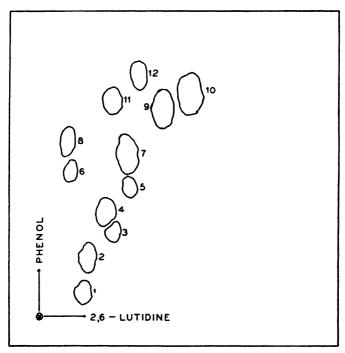


Fig. 1. Two-dimensional chromatogram of a chloride-free acid digest of yeast extract. The sample was applied at the lower left-hand corner and the solvents were allowed to move in the directions indicated, to the edges of the paper, as represented by the black lines in the figure. The phenol was saturated with water containing 10 per cent sodium citrate. (The use of sodium citrate was recommended by Helen Kirby Berry of this laboratory, to prevent distortion of the lower half of the developed chromatograms due to impurities in the phenol and possibly the filter paper.) The 2,6-lutidine contained water (65:35). Aspartic acid, 1; glutamic acid, 2; serine, 5; glycine, 4; threonine, δ ; lysine, δ ; alanine, δ ; arginine, δ ; valine, δ ; leucine or isoleucine, 10; "unknown" amino acid, 11; proline, 12. The position of 11 was unchanged by prolonged acid hydrolysis of yeast extract.

arating the "unknown" amino acid from the other amino acids present in the acid digest of yeast extract. n-Butanol-acetic acid-water 4:1:1 seemed best suited for this purpose (Fig. 2).

Partition Chromatography on Starch—To 10 ml. of the chloride-free acid digest of veast extract (containing approximately 1.13 gm. of solids)

were added 2 to 3 gm. of potato starch,³ and the mixture was lyophilized. To the dry powder were added several ml. of solvent to form a slurry and this was transferred to the top of a starch column (21×420 mm.) prepared in the usual manner from 115 gm. of starch. The column, with a solvent reservoir, was mounted on a Technicon automatic fraction collector,⁴ and the effluent was collected in 5 ml. fractions. Separation of the amino

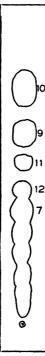


Fig. 2. One-dimensional chromatogram of a chloride-free acid digest of yeast extract. The sample was applied near the bottom of the paper and the solvent system (4:1:1 n-butanol-acetic acid-water) was allowed to move up the paper. The numbering of the amino acids is the same as in Fig. 1.

acids on the column was traced by paper partition chromatography of samples of each fraction of the effluent. The order of emergence of the amino acids from the column was the same as the order of the band rates on paper chromatograms (Fig. 2). The unknown amino acid began to emerge from the column after approximately 250 ml. of effluent had been collected. The chromatogram was stopped when most of the alanine had

- ² Obtained from the Fisher Scientific Company, Pittsburgh, Pennsylvania.
- 4 Obtained from the Technicon Company, New York.

emerged from the column. All fractions containing the unknown amino acid, some of which were contaminated with valine and others with proline and alanine, were combined and evaporated to dryness in vacuo. The residue was dissolved in water and rechromatographed on a separate column according to the directions described above. From this second chromatogram, several fractions were obtained which contained the amino acid uncontaminated by other amino acids, as demonstrated by paper chromatography.

Isolation of Amino Acid—Five individual starch chromatograms were prepared and developed according to the procedure described above. All fractions containing the unknown amino acid were combined, evaporated to dryness in vacuo, and the residue was rechromatographed on a single starch column. Those fractions containing only the unknown amino acid, as revealed by paper chromatography, were combined and evaporated to dryness in vacuo. The gummy residue (about 90 mg.) was dissolved in 2 to 3 ml. of water and the solution was decolorized with Darco G-60 and evaporated to dryness in vacuo. The residue was dissolved in 2 drops of water and 2 to 3 ml. of hot ethyl alcohol were added. Crystals separated spontaneously from the solution. After two more recrystallizations, 11 mg. of crystals were obtained; m.p. 192–195° (uncorrected).

Identification of Amino Acid—The crystalline sample was chromatographically pure. It possessed migration characteristics on paper chromatograms which were different from those of the known naturally occurring amino acids and a number of synthetic amino acids. Hydrolytic treatment did not affect the amino acid, as revealed by paper chromatography. The neutral equivalent obtained by electrometric titration was 102; N, 13.33 per cent. These data correspond to the empirical formula $C_4H_9O_2N$, an aminobutyric acid; N, calculated, 13.58; neutral equivalent, 103.1.

The infra-red absorption spectrum of the isolated amino acid was markedly different from that of α -amino-n-butyric acid, α -aminoisobutyric acid and β -amino-n-butyric acid as shown in Fig. 3. An authentic sample of γ -aminobutyric acid, m.p. 193–196° (uncorrected), was prepared according to the directions given in "Organic syntheses" (8). The hydrochloride of this sample melted at 132–133° (uncorrected). Abderhalden and Kautzsch report a melting point of 135–136° (9). The benzoyl derivative melted at 79° (uncorrected). The melting point reported by Kanewskaja (10) is 75°; N 6.53 per cent; theoretical 6.76 per cent. The benzoyl derivative was converted to N-benzoylpyrrolidone, m.p. 91–92° (uncorrected). An identical value was reported by Kanewskaja (10).

A comparison of the infra-red spectrum of the synthetic sample with

⁵ These are micro melting points.

⁶ These three compounds were Eastman Kodak White Label products.

L. J. REED 455

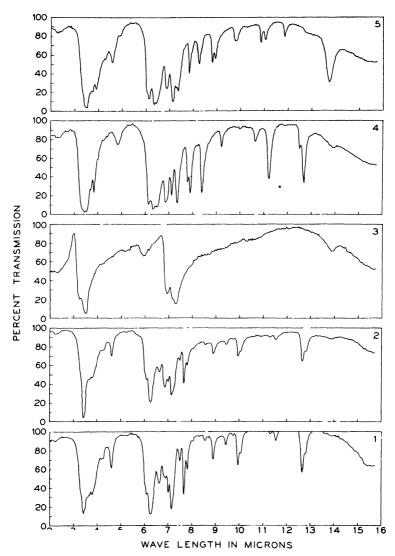


Fig. 3. Infra red absorption spectra of isomeric aminobutyric acids. The samples were mulled in mineral oil. Spectra 1 and 2, natural and synthetic γ -aminobutyric acids, respectively; Spectrum 3, α -amino-n-butyric acid; Spectrum 4, α -aminoisobutyric acid; Spectrum 5, β -amino-n-butyric acid.

that of the natural amino acid indicated that the two compounds are identical (Fig. 3). Confirmation of this identity was obtained by comparing the x-ray diffraction patterns of the two samples (Fig. 4). Chromatographic evidence demonstrated further that the isolated amino acid

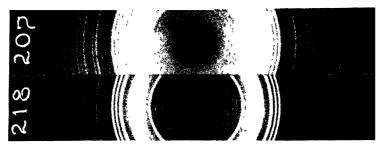


Fig. 4 \times ray diffraction patterns of natural (Sample 207) and synthetic (Sample 218) $_{\gamma}$ aminobutyric acids

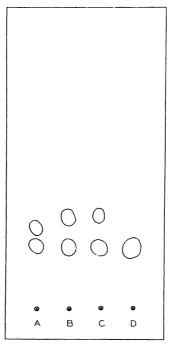


Fig. 5. One-dimensional chromatograms of mixtures of natural γ -aminobutyric acid and synthetic animobutyric acids. Mixtures of the natural amino acid (3 γ) and each of the following synthetic aminobutyric acids were applied near the bottom of the paper: A, β aminoisobutyric acid (4 γ) (prepared originally by Pollack (11) in this laboratory); B, α -amino-n-butyric acid (3 γ); C, α -aminoisobutyric acid (4 γ); D, γ -aminobutyric acid (3 γ). The solvent system was 2.6-lutidine-water. The lower spot in each case is the natural γ -aminobutyric acid.

and the synthetic γ -aminobutyric acid are identical (Fig. 5). β -Aminobutyric acid did not give a spot when tested in amounts up to 20 γ . Chro-

matograms developed with other solvent systems (phenol-water, n-butanol-acetic acid-water, isobutyric acid-water) revealed only a single spot when the sample chromatographed consisted of a mixture of the isolated amino acid and synthetic γ -aminobutyric acid.

Estimation of Amount of γ -Aminobutyric Acid in Yeast Extract—Further chromatographic experiments revealed that γ -aminobutyric acid is present in the free state in yeast extract (Difco). The amount of this amino acid was estimated by application of the method described recently by Bull and coworkers (12) for the quantitative determination of amino acids by filter paper chromatography. Known amounts of yeast extract, of synthetic γ -aminobutyric acid, and of mixtures of the two were run on a single large chromatogram with n-butanol-acetic acid-water as the solvent system (Fig. 2). The chromatogram was developed with ninhydrin, and color intensities were measured by means of a densitometer.\(^7\) It was estimated that yeast extract (Difco) contains 1 to 2 per cent by weight of γ -aminobutyric acid.

DISCUSSION

The present investigation establishes the occurrence of γ -aminobutyric acid in yeast extract. Since completion of this work, chromatographic evidence has been presented (13) which indicates that γ -aminobutyric acid is probably present in traces in blood and urine and in potato extracts. It has been recognized for many years that this amino acid is produced by bacterial action on glutamic acid (14). The glutamic acid decarboxylase involved in this reaction has not been demonstrated yet to occur in other living cells.

Studies in this laboratory with yeast indicate that γ -aminobutyric acid is a degradative product. It was found to be absent in freshly harvested yeast, but present in the free state in whole yeast which had been refrigerated for unknown periods of time, and in autolysates of yeast. These studies are being continued and will be reported at a later date.

STIMMARY

Partition chromatography on paper indicated the presence of an amino acid of unknown nature in yeast extract (Difco) which had been subjected to mild acid hydrolysis. This amino acid was isolated from the acid-treated yeast extract and identified as γ -aminobutyric acid. It was demonstrated later to be present in the free state in untreated yeast extract to the extent of 1 to 2 per cent by dry weight.

⁷ Obtained from the Photovolt Corporation, New York.

The author is grateful to Mr. W. L. Brown for the elementary analysis and neutral equivalent; to Dr. S. F. Kern for the x-ray diffraction data; to Mr. T. V. Parke for the infra-red absorption data; and to Dr. E. Bottorff for the preparation of the authentic sample of γ -aminobutyric acid, all of Eli Lilly and Company. The technical assistance of Miss Patricia Johnston is gratefully acknowledged.

BIBLIOGRAPHY

- 1. Consden, R., Gordon, A. H., and Martin, A. J. P., Biochem. J., 38, 224 (1944).
- 2. Dent, C. E., Biochem. J., 41, 240 (1947).
- 3. Dent, C. E., Stepka, W., and Steward, F. C., Nature, 160, 682 (1947).
- 4. Polson, A., Nature, 161, 351 (1948).
- 5. Ackermann, W. W., and Kirby, H., J. Biol. Chem., 175, 483 (1948).
- 6. Moore, S., and Stein, W. H., Ann. New York Acad. Sc., 49, 265 (1948).
- 7. Williams, R. J., and Kirby, H., Science, 107, 481 (1948).
- 8. Org. Syntheses, coll. 2, 25 (1943).
- 9. Abderhalden, E., and Kautzsch, K., Z. physiol. Chem., 81, 294 (1912).
- 10. Kanewskaja, S. J., Ber. chem. Ges., 69, 266 (1936).
- 11. Pollack, M. A., J. Am. Chem. Soc., 65, 1335 (1943).
- 12. Bull, H. B., Hahn, J. W., and Baptist, V. H., J. Am. Chem. Soc., 71, 550 (1949).
- 13. Dent, C. E., Biochem. J., 43, 169 (1948).
- 14. Gale, E. F., Advances in Enzymol., 6, 1 (1946).

DISTRIBUTION OF SELENIUM IN SERUM PROTEINS AND RED BLOOD CELLS AFTER SUBCUTANEOUS INJECTION OF SODIUM SELENATE CONTAINING RADIOSELENIUM

BY KENNETH P. McCONNELL AND BETTY J. COOPER

(From the Department of Radiation Biology, School of Medicine and Dentistry,

The University of Rochester, Rochester, New York)

(Received for publication, October 3, 1949)

In 1938, Smith, Westfall, and Stohlman (1) demonstrated that following prolonged feeding of naturally occurring seleniferous grains selenium is deposited in the body tissues, apparently in combination with the tissue proteins. Later work by Westfall and Smith (2), in which the distribution of selenium in liver and plasma of chronically poisoned animals was studied, showed that the element was present in all the proteins examined, though predominantly in the globulins. The manner in which the selenium in animal tissues is chemically bound is not known. A possible hypothesis is that selenium displaces the amino acid sulfur, or that its fixation involves the binding of sulfhydryl groups (3).

In earlier time-distribution studies the author observed a definite retention of trace amounts of radioselenium in the red blood cells and plasma of rats after subcutaneous injections of sodium selenate (4). The form in which the selenium enters the body apparently has little to do with its fixation.

In the experiments presented here it was planned to study the qualitative and quantitative distribution of selenium in the various blood proteins and in red blood cells. Following subcutaneous injection of sodium selenate containing trace amounts of radioselenium, the fixation of selenium in the proteins of plasma and in the hemin and globin fractions of hemoglobin was observed.

Procedure

Four normal dogs weighing 7.3 to 9.1 kilos were injected subcutaneously three times at 24 hour intervals with sublethal amounts of sodium selenate containing radioselenium. Previous to the second and third injections, and 24 hours after the last injections, samples of blood were taken from the carotid artery. Hematocrit and routine hematological studies were made. The red blood cells and plasma were separated by centrifugation, and the cells were washed twice with isotonic saline. Radioactivity measurements were made on samples of whole blood, plasma, and packed red blood cells, by use of a bell type Geiger-Müller tube.

The radioselenium was produced at Oak Ridge, Tennessee, by bombarding selenium powder with neutrons (5). The equation for the formation of radioselenium is as follows:

$$Se_{34}^{74} + N_0^1 \rightarrow Se_{34}^{75} + \gamma$$

Se⁷⁵ has a half life of 115 \pm 5 days which decays through K electron capture, emitting a probable 0.4 m.e.v. γ -ray, with the formation of stable As⁷⁵₃₃ (6).

The radioselenium was purified and sodium selenate was synthesized by methods described in a previous publication (4). Total selenium and radioactivity were determined on a sample of sodium selenate containing radioselenium. It was found that the sample contained 263 c.p.m. per microgram of selenium. This value was used throughout the experiments. A decay curve was constructed which showed the presence of a single isotope having a half life similar to that reported by Friedlander et al. (6).

For the purpose of studying the distribution of selenium in the various serum proteins, samples of blood were taken from the dogs 24 hours after the last injection. Total proteins were precipitated with 2.5 per cent trichloroacetic acid. Fractionation of the various serum proteins was carried out by the method of Majoor (7) as modified by Milne (8). Total globulins were precipitated from albumin with 26.8 per cent sodium sulfate solution, and euglobulins were separated from pseudoglobulins and albumin by use of 19.6 per cent sodium sulfate solution. The activity in the protein precipitates was measured, and the protein content of the precipitates and filtrates was determined by Clark's micro-Kjeldahl method (9). Selenium activity in the pseudoglobulin fractions was determined by subtracting that in the euglobulins from that in the total globulins, and the activity in albumin was determined by subtracting that in the globulins from that in the total protein.

Hemoglobin and its hemin and globin fractions were studied to determine where the selenium was located in the red blood cells. Crystalline hemoglobin was isolated from the red blood cells of three dogs that had been injected with sodium selenate containing radioselenium, by use of the method of Welker and Williamson (10). The crystalline hemoglobin was recrystallized, and it was found that photomicrographs of the crystals compared favorably with those reported by Reichert and Brown (11). Crystalline hemin was isolated by the method of Nencki and Zaleski (12). The crystals were washed with 50 per cent acetic acid, 95 per cent alcohol, and ether. The globin fraction was studied following treatment of crystalline hemoglobin with acetone and hydrochloric acid after the method of Anson and Mirsky (13). The globin was dissolved in chilled 0.1 N hydrochloric acid and precipitated with acid acetone three times.

The various blood fractions were prepared for measurements of radioactivity by wet ashing with concentrated nitric acid and hydrogen peroxide. Digests were made to a 10 cc. volume, and 1 cc. aliquots were taken for counts.

A recovery study was made. The average counts in Table I with the standard error of 8.25 show no evidence that the presence of liver and bone tissue affects the determination of the activity of the standard. The average net count represents the average count per minute (minus background) obtained from two 5 minute determinations. Values for the original standard were obtained from an aliquot of sodium selenate containing radioselenium. For a control, an equivalent amount of radioselenium was added to the amounts of HNO₃ and H₂O₂ that were used for ashing tissues and was then carried through all the experimental steps. An equivalent

Table I

Recovery of Radioselenium Added to Tissues
Standard error of each average count 8.25 counts per minute.

	Tissue						
Origin	197.3						
Control (with all steps through reagents identical)							199.4
						rd	193.0
1 "	"	" +	"	"	"		224.0
2 ''	**	" +	"	"			190.4
2 ''	44	" +	"	"	4.6		194.9
1 "		bone +	"	"	44		205.1
911	"	" +	"	"	"		207.7

amount of radioselenium was then added to various tissues which were wet ashed and the radioactivity determined.

RESULTS AND DISCUSSION

The data obtained in these experiments are presented below in three sections, dealing with the distribution of selenium (1) in the plasma and red blood cells, (2) in the various serum protein fractions, and (3) in the hemoglobin of the red blood cells.

Selenium in Plasma and Red Blood Cells—The distribution of selenium in dog plasma and red blood cells after repeated injections of sodium selenate containing radioselenium is shown in Fig. 1. The results are expressed as counts per 100 cc. of whole blood. The activity values for plasma and red blood cells were obtained by multiplying the number of counts for 100 cc. of plasma and red blood cells by the corresponding hematocrit value.

It will be noted that the concentrations of selenium in whole blood and

in the red blood cells increase appreciably with time, and that the concentration is greater in the red blood cells than in the plasma. It appears that the concentration of selenium in the plasma when determined at 24 hour intervals varies within very narrow limits throughout the experiment.

Samples of blood were taken from Dog A 24 hours after the last injection, as well as at 48 and 144 hour periods. The results show that the selenium content of whole blood, red blood cells, and plasma decreases with time, the

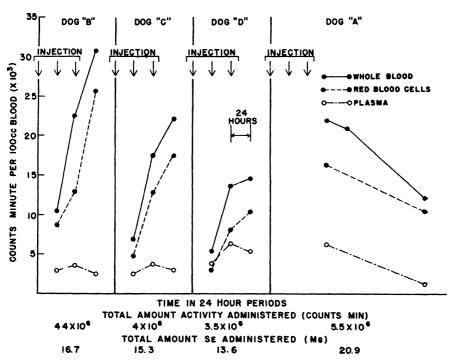


Fig. 1. Distribution of selenium in dog plasma and red blood cells after repeated subcutaneous injection of sodium selenate containing radioselenium.

plasma values falling off rapidly to a very low level. The level for the red blood cells decreases but remains relatively high in relation to the plasma.

It was of interest to determine the per cent of whole blood activity in red blood cells and plasma 24 hours after the last of three injections. The activity in the red blood cells was found to be 74, 91, 80, and 72 per cent (average of 79) for Dogs A, B, C, and D respectively. The activity in plasma was 26, 8, 14, and 39 per cent (average of 22), respectively.

By use of the value of 7 per cent of body weight as the total circulating blood (14), it is possible to calculate the per cent of the total selenium administered in the total circulating blood 24 hours after the last of three

injections. The results are as follows: Dog A 2.5 per cent, Dog B 3.9 per cent, Dog C 3.4 per cent, and Dog D 2.0 per cent.

It is apparent from the data reported here that selenium is concentrated to a greater extent in the red blood cells than in the plasma, and that 2.0 to 3.9 per cent of the total selenium administered is found in the total circulating blood.

Selenium in Serum Proteins—In the next series of experiments, the distribution of selenium in the various serum protein fractions was studied. Table II shows the fractionation of serum proteins of three dogs into their various components; *i.e.*, total globulin, albumin, euglobulin, and pseudoglobulin. The selenium content in total protein ranges from 5.4 to $10.2 \, \gamma$ per gm. of protein. It will be noted that selenium is not found in

Table II

Distribution of Selenium in Various Protein Fractions of Dog Serum after Subcutaneous Administration of Sodium Selenate

t.	Do			og B	Dog D		
Fraction	Counts per min. per gm. protein	Se per gm. protein	Counts per min. per gm. protein	Se per gm. protein	Counts per min. per gm. protein	Se per gm. protein	
		γ		γ		γ	
Total protein	2685	10.2	2278	8.7	1418	5.4	
Globulin	2563	9.7	2614	9.9	1463	5.6	
Albumin	3590	13.6	1672	6.4	1250	4.8	
Euglobulin	1360	5.2	2434	9.2	1558	5.9	
Pseudoglobulin	3530	13.4	3371	12.8	1255	4.8	

any one fraction, but is present in all of the protein fractions examined, and the amount in each of the various fractions is in general similar but not identical. It appears that the fixation of selenium occurs to about the same extent in the globulin and albumin fractions, since the amount of globulin and albumin selenium with respect to total protein selenium for the three dogs was found to be 54 ± 10 and 45 ± 11 per cent, respectively.

The question arises whether the affinity of protein for selenium results in a definite fixation in the protein matrix or whether selenium in the protein fraction is due to adsorption on the surface of the protein molecule. The following information tends to support the contention that selenium occupies a well defined position in the protein molecule: Serum protein selenium in dilute solution resists thorough dialysis against neutral, acidic, and basic solutions. A sample of total serum protein was precipitated, and the precipitate washed thoroughly, dissolved in isotonic saline, and

divided into four equal aliquots. One aliquot served as a control and was analyzed for total protein and radioselenium. The remaining aliquots were dialyzed against buffered solutions maintained at pH 2.8, 7, and 9, after which protein and selenium determinations were made. The results reveal that the selenium was not dialyzed under the above conditions (Table III).

The results indicate that the selenium present in the blood proteins is in some organoselenium complex. In this regard it is of interest to compare the metabolism of inorganic selenium with that of inorganic sulfur. It has been shown that inorganic sulfur can be converted to organic sulfur. Smythe and Halliday (15) were able to demonstrate the formation of cysteine from sulfide sulfur in the presence of a suitable liver enzyme. In another study, Dziewiatkowski (16) was successful in showing that a small fraction of the radioactive sulfur administered to rats as sulfide was

Table III

Dialysis of Dog Serum Proteins at Various pH Values
Clark and Lubs buffer mixtures were used.

Experiment	Se per gm. protein
	γ
Control	3.4
pH 2.8	4.0*
" 7.0	3.3*
" 9.0	3.8

^{*} Corrections made for potassium activities.

incorporated in the cystine isolated from hair, liver, skeletal muscle, and skin. More recently, Dziewiatkowski (17) reports that labeled S³⁶-sodium sulfate, when administered to suckling rats, was found to be present in the articular cartilage as chondroitin sulfate. As we have proposed in an earlier report (18), a similar metabolic process may take place with selenium, as with sulfur, in the conversion of inorganic selenium to some organoselenium protein complex. Whether selenium displaces the amino acid sulfur or binds sulfhydryl groups or combines with an available radical cannot be stated at this time.

Selenium Activity in Hemoglobin, Hemin, and Globin—In the study on the distribution of selenium in the various components of the red blood cell, hemoglobin, hemin, and globin were isolated and analyzed separately for selenium activity. Data obtained from these studies are presented in Table IV, where weighed samples of crystalline hemoglobin and hemin, and of globin were found to have 4.5 to 5.7, 10.6 to 15.5, and 1.3 to 2.4 γ of selenium per gm. respectively. Activity ratios of hemin to globin on the

gm. basis for two dogs were found to be 6.3 and 8.1. It is apparent from the data presented here that selenium is present in hemoglobin, and that the amount of selenium in the hemin fraction when calculated on the gm. basis is greater than that in the globin fraction.

The fixation of selenium in red blood cells presumably does not take place in bone marrow, for we were unable to detect any significant selenium activity in bone marrow 24 hours after the last of three injections. The incorporation of selenium in hemoglobin may take place by exchange with some normally occurring substance, or by addition to an available radical after diffusion across the cell membrane. Inorganic selenium is quite unlike radioactive iron in that iron is synthesized into hemin in the bone marrow (19). Of particular interest is the apparent difference between selenium and sulfur in their ability to concentrate in the bone marrow.

Table IV
Selenium Activity of Hemoglobin, Hemin, and Globin in Dogs after Subcutaneous
Administration of Sodium Selenate

•	Activity of	f hemoglobin	Activity	of hemin	Activity of globin		
Dog	Counts per min. per gm. Hb	Se per gm. Hb	Counts per min. per gm. hemin	Se per gm. hemin	Counts per min. per gm. globin	Se per gm. globin	Ratio, hemin-globin, gm. basis
		γ		γ		γ	
В	1449	5.50	4082	15.5	644	2.4	6.3
	1506	5.72	4016	15.3			
\mathbf{C}	1235	4.69	2786	10.6	346	1.3	8.1
	1329	5.05		1			
A	1225	4.65		1 1			
	1184	4.50					

Singher and Marinelli (20) as well as Dziewiatkowski (21) were able to demonstrate, using labeled sodium sulfate, that S^{26} was found in the bone marrow, while we were unable to detect any selenium activity in dog bone marrow or in rat bone (4) after administering labeled sodium selenate. The destruction and elimination of selenium-bound hemoglobin may take place, in part, in the liver, for we have observed the selenium content in bile 24 hours after the last of three injections in three dogs to be 4.2, 8.6, and 4.3 γ of selenium per cc. of bile.

SUMMARY

Studies were made on the distribution of selenium in the blood, serum proteins, and red blood cells of the dog after subcutaneous injections of radioselenium as sodium selenate.

1. It was found 24 hours after the last of three injections that the whole

blood activity in red blood cells for four dogs averaged 79 per cent (72 to 91 per cent), while in plasma it averaged 22 per cent (8 to 39 per cent). It was calculated that 2.0 to 3.9 per cent of the selenium administered was present in the total circulating blood.

- 2. The results obtained from serum protein fractionation revealed that selenium was present in albumin, globulin, euglobulin, and pseudoglobulin, and that the amount of selenium in the various fractions when expressed as micrograms of selenium per gm. of protein was similar but not identical. Results from dialysis studies indicated that there was a certain amount of selenium fixation in blood proteins which existed in a fairly stable protein-like combination.
- 3. It has been shown that selenium is present in crystalline hemoglobin and hemin and in globin. The amounts of selenium in hemin when calculated on the gm. basis are greater than in globin.

The authors wish to express thanks to Dr. Joe W. Howland for his interest in this investigation.

BIBLIOGRAPHY

- Smith, M. I., Westfall, B. B., and Stohlman, E. F., Pub. Health Rep., U. S. P. H. S., 53, 1199 (1938).
- Westfall, B. B., and Smith, M. I., Pub. Health Rep., U. S. P. H. S., 55, 1575 (1940).
- 3. Painter, E. P., Chem. Rev., 28, 179 (1941).
- 4. McConnell, K. P., J. Biol. Chem., 141, 427 (1941).
- 5. Radioisotopes, United States Atomic Energy Commission, Oak Ridge (1947).
- 6. Friedlander, H. N., Seren, L., and Turkel, S. H., Phys. Rev., 72, 23 (1947).
- Majoor, C. L. H., Thesis, Amsterdam (1942); Yale J. Biol. and Med., 18, 419 (1946).
- 8. Milne, J., J. Biol. Chem., 169, 595 (1947).
- 9. Clark, E. P., J. Assoc. Offic. Agr. Chemists, 24, 641 (1941).
- 10. Welker, W. H., and Williamson, C. J., J. Biol. Chem., 41, 75 (1920).
- Reichert, E. T., and Brown, A. P., The crystallography of hemoglobins, Washington (1909).
- 12. Nencki, M., and Zaleski, J., Z. physiol. Chem., 30, 384 (1900).
- 13. Anson, M. L., and Mirsky, A. E., J. Gen. Physiol., 13, 469 (1930).
- 14. Skelton, H., Arch. Int. Med., 40, 140 (1927).
- 15. Smythe, C. V., and Halliday, D., J. Biol. Chem., 144, 237 (1942).
- 16. Dziewiatkowski, D. D., J. Biol. Chem., 164, 165 (1946).
- Dziewiatkowski, D. D., Benesch, R. E., and Benesch, R., J. Biol. Chem., 178, 931 (1949).
- 18. McConnell, K. P., J. Biol. Chem., 173, 653 (1948).
- 19. Copp, D. H., and Greenberg, D. M., J. Biol. Chem., 164, 377, 389 (1946).
- 20. Singher, H. O., and Marinelli, L., Science, 101, 414 (1945).
- 21. Dziewiatkowski, D. D., J. Biol. Chem., 178, 197 (1949).

BIOLOGICAL ACTIVITY OF PURE VITAMIN A2*

By EDGAR M. SHANTZ AND JOHN H. BRINKMAN

(From the Research Laboratories of Distillation Products, Inc., Rochester, New York)

(Received for publication, October 6, 1949)

The degree of biological activity for mammals that can be attributed to vitamin A2 has long been a matter of disagreement among various investigators. Karrer et al. (1) first reported that vitamin A2 possessed no vitamin activity for mammals. This conclusion, however, was based on the difficult procedure of attempting to evaluate a mixture of vitamins A1 and A2 biologically. In a later paper (2) this same group claimed that vitamin A₂ had one-tenth the growth-promoting power of vitamin A₁ for rats and ascribed this activity to the ability of the animals to convert a small portion of the vitamin A₂ to vitamin A₁. These observations were based on biological tests carried out by Professor H. von Euler in Stockholm. On the other hand, Gillam et al. (3) believed that vitamin A₂ possessed a considerable degree of biological activity and showed (4) that vitamin A₂ was stored in the livers of animals receiving vitamin A₂ in their diet. Lederer and Rathmann (5) also demonstrated that vitamin A₂ could be stored in the livers of rats and frogs but the degree of biological activity was not deter-From a study of the vitamin A₁ and A₂ distribution in various fish and sea birds, Lovern et al. (6) concluded that vitamin A2 does not replace vitamin A₁ with equal readiness for all functions.

Rosanova (7) states that vitamin A_2 has an "activating effect" upon the growth of rats and is a factor in their vision. Shantz *et al.* (8) found that in rats fed vitamin A_2 rhodopsin in the retinas was replaced by porphyropsin, and from this and other observations concluded that vitamin A_2 could replace vitamin A_1 in all physiological functions. Using human subjects, Millard and McCann (9) have shown that the threshold of rod sensitivity to red light can be lowered slightly by administration of vitamin A_2 over a period of 16 weeks.

In an earlier paper from this laboratory (10), a vitamin A_2 concentrate was reported to have a potency of 47,500 U. S.P. units per gm. Various reviewers have mistakenly reported that this potency referred to pure vitamin A_2 rather than to a concentrate of unknown strength (11-13). Actually, since the $E_{1\text{cm}}^{1\%}$ (351 m μ) value of the concentrate equaled 39.0, the conversion factor for vitamin A_2 could be calculated as 47,500 \div 39 =

^{*} Presented before the Division of Biological Chemistry at the meeting of the American Chemical Society at Washington, D. C., September, 1948. Communication No. 159 from the Laboratories of Distillation Products, Inc.

1218, but no estimate could be made of the potency of pure vitamin A_2 because its $E_{1 \text{ cm.}}^{1\%}$ (351 m μ) value was not known.

The recently reported isolation of pure vitamin A_2 (14) in this laboratory and the acceptance of the new and stable oil solution of crystalline vitamin A acetate as the U. S. P. reference standard (15) have made possible a more accurate determination of the true biological potency of vitamin A_2 .

EXPERIMENTAL

Biological Assay—The pure vitamin A_2 alcohol was biologically assayed by essentially the method of the United States Pharmacopeia XIII (15). The crystalline p-phenylazobenzoate of vitamin A_2 was also assayed. Two levels of vitamin A acetate reference oil, two levels of vitamin A_2 alcohol, and one level of vitamin A_2 p-phenylazobenzoate were fed to the rats. The growth response for each of these levels is shown in Table I.

Table I

Growth Response Produced by Vitamin A_2

No. of rats	Material fed	Amount fed	Weight gain in 28 days
		U. S. P. units	gm.
7	Vitamin A acetate reference standard	1.2	35.7
7	., ., ., ., .,	2.4	73.0
		mg.	
5	" A ₂ alcohol	0.65	21.0
7		1.30	52.1
7	" p-phenylazobenzoate	2.3	52.1

Curves of log dose *versus* growth response were plotted for the two levels of reference standard and the two levels of vitamin A_2 alcohol. Calculation by factorial analysis of variance according to Bliss and Marks (16) showed that each of the dosage levels of vitamin A_2 was equivalent to 70.0 ± 8.0 per cent of its corresponding level of reference standard. Thus, by this assay, vitamin A_2 was found to have a biological potency of 1,300,000 U. S. P. units per gm. and the true value probably does not deviate more than 100,000 units from this figure. Vitamin A_2 , therefore, has approximately 40 per cent of the activity of crystalline vitamin A_1 .

The vitamin A_2 p-phenylazobenzoate, which was fed at only one level, gave exactly the same growth response as the higher level of vitamin A_2 alcohol. Its potency was calculated to be 730,000 U. S. P. units per gm. for the crystalline ester. When only the vitamin moiety of this compound is considered, a value of 1,270,000 U. S. P. units per gm. is obtained, which is in excellent agreement with the value found for the free alcohol.

Conversion Factors—It is common practice to evaluate the potency of an oil containing vitamin A by multiplying its ultraviolet extinction coefficient by a conversion factor. From the above biological potency and the value of 1460 reported (14) as the extinction coefficient of pure vitamin A_2 alcohol at its absorption maximum at 351 m μ , it can be calculated that this compound has a conversion factor of approximately 900 when measured at this wave-length.

The extinction coefficient of vitamin A_2 in the vitamin A_1 region at 325 m μ is 80 per cent of its peak value, since its absorption curve is rather broad (14). At 325 m μ , therefore, the conversion factor for vitamin A_2 is 900 ÷ 0.8 = 1125. Thus, if vitamin A_2 happens to be an unsuspected component of a fish liver oil, the true biological potency of the oil will tend to be slightly lower than that calculated from the observed spectrophotometric value at 325 m μ , with the currently accepted conversion factors of 1894 for vitamin A_1 .

The broad overlapping of the vitamin A_1 and A_2 spectral curves (14) makes measurement of ultraviolet absorption a rather poor method of determining the relative quantities of each in mixtures. Morton and Stubbs (17–19) have proposed a correction procedure whereby it is claimed that the true E value of vitamin A in an oil can be determined by subtraction of the irrelevant absorption due to extraneous material. The distorting effect of interfering substances is calculated from the interrelationships of three reference points. These reference points are obtained from the absorption curve of pure vitamin A in a given solvent and occur at those wave-lengths at which the absorption is at a maximum and on either side when the absorption is 6/7 maximal. For the derivation of the formulas and their geometric basis, the reader is referred to the original papers.

The above correction method is based on two premises: first, that substances other than vitamin A have a linear absorption between the two "fixation points" on either side of the maximum, and second, that these substances have no vitamin A activity of their own. Neither of these assumptions holds true in the case of vitamin A₂.

Oser (20) has combined and simplified the correction factors of Morton and Stubbs into the single equation

$$f = 7 - 7a \frac{E_x}{E_m} - 7(1 - a) \frac{E_y}{E_m}$$

where f is the factor by which the observed E_m is multiplied to obtain the corrected value; E_m , E_x , and E_y are the observed extinction values at the wave-lengths m, x, and y respectively; and m, x, and y are those wavelengths on the absorption curve of pure vitamin A_1 at which the maximum

and the two "fixation points" below and above the maximum occur. The value a = (y - m)/(y - x).

According to Oser (20) the values m, x, and y for vitamin A alcohol in ethanol solution are 325, 311, and 335.5 m μ , respectively, and the value thus obtained for 7a=3.0 and for 7(1-a)=4.0. From an absorption curve of pure vitamin A_2 alcohol in ethanol solution, we have determined in this laboratory that the extinction ratio E (311 m μ) to E (325 m μ) = 0.69, and the ratio E (335.5 m μ) to E (325 m μ) = 1.15. By substituting in the above equation, it can be calculated that f=0.33 when pure vitamin A_2 is measured as vitamin A_1 alcohol at 325 m μ , corrected by the Morton-Stubbs procedure. By this method, then, an oil which contains a significant amount of vitamin A_2 will tend to be overcorrected because that portion of the 325 m μ absorption which is contributed by vitamin A_2 will in effect be measured with a conversion factor of 0.33 \times 1894 = 625

Material fed	Total amount per rat stored in liver	Per cent total dose stored	
	U. S. P. units		
Vitamin A ₁ alcohol	491	32.7	
" " acetate	690	46.0	
" A2 alcohol	275	18.3	
" " acetate	345	23.0	

instead of the true factor of 1125. Such an error might be appreciable in certain commercial fish liver oils, which have been found to contain as much as 10 per cent of the vitamin A in the form of vitamin A_2 (19). In such a case the vitamin A_2 content of the oil might best be determined independently by calculation from the blue color absorption obtained with antimony trichloride with the E(620) to E(690) ratios suggested by Jensen ct al. (10), or by the procedure of Embree and Shantz (21) in which the two anhydro derivatives are chromatographically separated.

Liver Storage Tests—Four groups of vitamin A-depleted rats were fed biologically equivalent doses of the compounds listed in Table II for a period of 3 days. The animals were killed on the 5th day and the total amount of vitamin A stored in the liver was determined in each case. From an examination of Table II, it can be seen that the storage of vitamin A_1 is better than that of vitamin A_2 , although within each group somewhat more of the acetate ester is stored than is the free alcohol. All of the test substances were diluted to approximately the same level (5000 U. S. P. units per gm.) in Wesson oil before feeding.

It is interesting to note that the liver storage ratios of vitamins A_1 and A_2 have roughly the same relationship as their biological potencies in the U. S. P. assay. However, it is difficult to say at the present time whether these findings are significant and related.

Non-Conversion of Vitamin A_2 in Vivo—Animals that were maintained on doses of vitamin A_2 close to the minimum daily maintenance level did not store enough vitamin in the liver for colorimetric determination. However, an excellent curve for blue color could be obtained with antimony trichloride on the liver extracts from rats receiving about 50 U. S. P. units per day. The curves thus obtained were identical in shape with that of the pure vitamin A_2 originally fed. This confirms our earlier report (9) that vitamin A_2 is not converted to vitamin A_1 in the animal body.

SUMMARY

Pure vitamin A_2 alcohol has a potency of about 1,300,000 units per gm. as determined by the U. S. P. rat growth procedure. It is stored in the liver with an efficiency somewhat lower than that of vitamin A_1 , but does not appear to be converted to vitamin A_1 in vivo.

BIBLIOGRAPHY

- 1. Karrer, P., Geiger, A., and Bretscher, E., Helv. chim. acta, 24, 161 E (1941).
- 2. Karrer, P., and Bretscher, E., Helv. chim. acta, 26, 1758 (1943).
- Gillam, A. E., Heilbron, I. M., Jones, W. E., and Lederer, E., Biochem. J., 32, 405 (1938).
- 4. Gillam, A. E., Biochem. J., 32, 1496 (1938).
- 5. Lederer, E., and Rathmann, R., Biochem. J., 32, 1252 (1938).
- 6. Lovern, J. A., Morton, R. A., and Ireland, J., Biochem. J., 33, 325 (1939).
- 7. Rosanova, V. A., Khim. Referat. Zhur., 4, 55 (1941).
- Shantz, E. M., Embree, N. D., Hodge, H. C., and Wills, J. H., Jr., J. Biol. Chem., 163, 455 (1946).
- 9. Millard, E. B., Jr., and McCann, W. S., J. Appl. Physiol., 1, 807 (1949).
- Jensen, J. L., Shantz, E. M., Embree, N. D., Cawley, J. D., and Harris, P. L., J. Biol. Chem., 149, 473 (1943).
- 11. Chem. Abstr., 38, 404 (1944).
- 12. Nutr. Rev., 5, 17 (1947).
- 13. Gortner, R. A., Outlines of biochemistry, New York, 3rd edition, 889 (1949).
- 14. Shantz, E. M., Science, 108, 417 (1948).
- The Pharmacopœia of the United States of America, thirteenth revision, Easton (1949).
- 16. Bliss, D. I., and Marks, H. P., Quart. J. Pharm. and Pharmacol., 12, 182 (1939).
- 17. Morton, R. A., and Stubbs, A. L., Analyst, 71, 348 (1946).
- 18. Morton, R. A., and Stubbs, A. L., Biochem. J., 41, 525 (1947).
- 19. Morton, R. A., and Stubbs, A. L., Biochem. J., 42, 195 (1948).
- 20. Oser, B. L., Anal. Chem., 21, 529 (1949).
- 21. Embree, N. D., and Shantz, E. M., J. Biol. Chem., 132, 619 (1940).

PARTIAL PURIFICATION OF A FACTOR ESSENTIAL FOR GROWTH OF LEUCONOSTOC CITROVORUM

By JOHN C. KERESZTESY AND MILTON SILVERMAN

(From the Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, October 18, 1949)

Based on the growth response of Leuconostoc citrovorum to commercial antianemia liver preparations, Sauberlich and Baumann (1) suggested that the organism might be responding to the antipernicious anemia principle. Since vitamin B₁₂ was available to us at that time, it was tested for its growth-promoting activity on this organism and was found to be negative. Efforts were then undertaken to concentrate and isolate the factor essential for the growth of this organism. Methods for the preparation of concentrates of an acid-labile factor and a description of some of its properties are now presented.

Methods

A culture of the organism, L. citrovorum (8081) was obtained from the American Type Culture Collection. It was maintained in yeast extract-glucose agar stabs and transferred in this medium at biweekly intervals. For assay, the organism from the stab culture was inoculated into a liver broth of the following composition: tryptone, Difco, 2 per cent; tryptose, Difco, 0.5 per cent; yeast extract, Difco, 0.5 per cent; tomato juice, 20 per cent; water-soluble liver (Wilson), 0.5 per cent; glucose, 0.3 per cent; lactose, 0.2 per cent; Tween 80, 0.005 per cent, pH 6.5.

After incubation for 24 hours at 37°, the culture was washed twice in NaCl solution (0.85 per cent) and diluted 10-fold. 1 drop of the washed suspension in saline was used to inoculate each assay tube.

The assay medium was essentially that of Snell, Kitay, and McNutt (2). It was modified by the omission of Tween 80 and oleic acid and by doubling the recommended casein hydrolysate concentration. Difco casamino acids, untreated by charcoal, were used as the source of casein hydrolysate.

The above basal medium (double strength) was employed in a volume of 5 ml. to which a total of 5 ml. of water and sample was added. The tubes were autoclaved for 10 minutes at 10 pounds steam pressure, then placed in a cold water bath, shaken to dissolve a precipitate which formed,

¹ Suggested by M. Rogosa.

and cooled to room temperature. The assay tubes were then inoculated and incubated for 15 to 16 hours at 37°. Turbidity measurements were then made by means of an Evelyn photelometer with the 660 m μ filter.

A hot aqueous extract of fresh kale, containing 7.4 mg. of solids per ml., was initially employed as a reference standard. Later, an ammonium salt of the active material obtained after chromatography was substituted for this extract. Results are expressed in growth equivalent to the activity of the kale extract and 1 unit was arbitrarily established as representing the activity of 1 ml. of this extract.

Folic acid determinations² were made in the medium of Teply and Elvehjem (3).

EXPERIMENTAL

4 kilos of liver fraction S (Wilson) were suspended in 340 liters of water. The pH of the suspension was adjusted to 5.5 with sodium hydroxide or sulfuric acid as required. The insolubles were removed by filtration.

To this extract were added 4.5 kilos of norit A (Pfanstiehl), and it was stirred for 30 minutes. The norit adsorbate was recovered by filtration and washed with water. About 10 per cent of the activity was lost in the filtrate from the norit.

The activity was recovered from the norit by extracting three times with the following mixture: 13.3 liters of 95 per cent ethyl alcohol, 26.6 liters of water, and 1 liter of 10 per cent sodium hydroxide. The extractions were made at room temperature, with 30 minutes stirring. The combined eluates contained approximately 65 per cent of the original activity.

Readsorption—The combined eluates were acidified to pH 4.5 with 50 per cent sulfuric acid and stirred for 30 minutes with 1.5 kilos of norit A. The recovered norit adsorbate was washed with 5 liters of 30 per cent ethyl alcohol. It was then extracted four times with 15 liters of 30 per cent ethyl alcohol to which were added 750 ml. of concentrated ammonium hydroxide. The extractions were made at room temperature with vigorous stirring for 30 minutes.

The combined cluates contained 50 to 60 per cent of the original activity, and represented a 20-fold purification.

Butanol Extraction—The combined eluates were concentrated at reduced pressure, temperature 45–50°, to approximately 1 liter. This was then acidified to pH 2.3 with 10 per cent nitric acid. The precipitates which had formed during the concentration and acidification were removed by filtration through a thin layer of Super-Cel. About 10 per cent of the

² In the text folic acid is referred to as FA and Streptococcus lactis R as SLR.

activity was retained in this precipitate. The acid filtrate was immediately extracted with approximately 12 liters of water-saturated butanol. The water phase was removed and contained about 15 per cent of the activity. The butanol layer was extracted five times with 8 liter portions of water. The aqueous extracts of the butanol which contained 30 to 35 per cent of the original activity were promptly neutralized with barium hydroxide and concentrated *in vacuo* at 45–50° to a volume of 150 to 200 ml. This concentrate was stored at 3–5° for several days, during which time a heavy precipitate formed which was removed and washed by centrifugation.

The clear solution was then acidified to pH 4.9 with 10 per cent nitric acid. Silver nitrate was added in excess. The precipitate was recovered by centrifugation and washed with water. The washed silver precipitate was suspended in excess dilute ammonia water, and then the silver was precipitated as the sulfide with hydrogen sulfide. The silver sulfide was washed several times with water. It was found necessary to use ammonium hydroxide to maintain alkaline conditions during the decomposition of the silver precipitate, since acidity resulting in the decomposition of the silver precipitate with hydrogen sulfide was sufficient to inactivate a substantial portion of the growth factor.

The filtrate and washings resulting from the decomposition of silver precipitate were concentrated *in vacuo*. Excess barium hydroxide was added and the extract was further concentrated to about 75 ml. The recovery to this stage was 25 to 35 per cent.

The concentrate was then stored for several days at 3-5°. The insoluble material which separated was discarded after washing with 30 to 40 ml. of water. The clear extract and wash were combined and 4 volumes of 95 per cent ethyl alcohol were added. This was set in the refrigerator overnight. The precipitated barium salts were recovered, washed with alcohol, acetone, and ether, and finally dried *in vacuo*.

At this stage the recovery of activity was 20 to 30 per cent. The potency of the barium salts varied from 70 to 300 units per mg., which corresponded to a 70- to 300-fold purification of starting liver fraction S.

Chromatography—To effect further purification the barium salts were chromatographed on aluminum oxide. The adsorptions were carried out in acid alcohol and elutions by dilute ammoniacal aqueous alcohol. A typical example is as follows: 14.42 gm. of a barium salt having a potency of 109 units per mg. were dissolved in 200 ml. of water. 10 per cent hydrochloric acid was added to pH 2.3. 3 volumes of 95 per cent ethyl alcohol were added. The precipitate which formed was separated by centrifugation and washed with 400 ml. of 75 per cent ethyl alcohol. The main solution and wash were combined and allowed to pass through

a 40 mm. column containing 300 gm. of aluminum oxide (Alcoa). The column was washed with 30 per cent ethyl alcohol and eluted with 30 per cent ethyl alcohol to which was added 1 per cent concentrated ammonia water. Fractions were taken as indicated in Table I.

Fraction No., 50 ml.	Activity	Potency	Fraction No., 50 ml.	Activity	Potency
	units	units per mg.		units	units per mg
7*	1,800	24	15	144,000	3200
8	12,600	210	16	117,000	3340
9	45,500	600	17	72,000	2400
10	99,000	1320	18	49,000	2450
11	166,500	2875	19	33,000	940
12	190,000	3450	20	29,500	1060
13	187,500	3410	21	26,000	650
14	158,000	3160			

Table I

Distribution of L. citrovorum Activity on Aluminum Oxide Column

Acid Lability and Other Properties of Concentrates—Concentrates active for the test organism show marked acid lability. The effect of storage for 6 hours at room temperature at several pH values is as follows:

pH, adjusted with								
$\mathrm{H}_2\mathrm{SO}_4\ldots$	1.12	1.30	2.20	3.05	3.60	4.18	4.72	5.0
Loss, %	100	100	81	30	13	0	0	0

Loss of growth activity is evidenced at pH 3.6. In 0.1 N H₂SO₄, 90 to 100 per cent destruction results in 24 hours at room temperature or in 15 minutes when the material is autoclaved at 10 pounds steam pressure. No destruction under such conditions is detectable when the active material is stored in 0.1 N NaOH.

The distribution of the L. citrovorum-active material between water and an equal volume of water-saturated normal butanol is dependent on pH. The effect of pH on the distribution is as follows:

pHActivity in butanol layer, %			2.30 48.5

An equal distribution of the activity between the two phases occurs at pH 2.2 to 2.3. Lyman and Prescott (4) by electrolytic procedures have also indicated the acidic properties of the *citrovorum* factor.

^{*} Fractions 1 through 6 contained 695 mg. of solids but no activity.

The active material is completely precipitated by heavy metals such as lead and silver. Only partial precipitation (40 to 50 per cent) occurs at pH 7.0 with zinc. At pH 2.3 the material is not extracted by ethyl ether or chloroform and is not precipitated from aqueous solution by ethanol or acetone. Esterification procedures (diazomethane or methanol and HCl) resulted in complete inactivation as indicated by lack of activity after alkaline hydrolysis. Acylation also resulted in inactivation.

Relationship of SLR to L. citrovorum Activity—Concentrates containing the citrovorum factor are also active in promoting the growth of S. lactis R and Lactobacillus casei in media lacking folic acid. This relationship is indicated for SLR in Table II, showing the activity of successive samples obtained during chromatography. The growth activity for SLR paralleled that for L. citrovorum. This is indicated by the relatively constant ratios shown.

iple No.	SLR*	Citrovorum	SLR	SLR after acid	Per cent residual SLR	
•			citrovorum	treatment	activity	
	mugm per ml.	units per ml.		mugm. per ml.		
1	200	35.2	5.7	108	54	
2	8,900	1480	6.0	4,800	54	
3	25,800	4800	5.4	13,950	54	
4	28,750	4900	5.9	15,000	52	
5	27,000	4920	5.5	13,950	52	
6	19,200	3330	5.7	11,800	59	

TABLE II
Ratios of SLR to L. citrovorum Activity

Acid treatment of these fractions also results in a rather consistent percentage loss in SLR growth activity (Table II). As indicated previously, the citrovorum factor is acid-labile and storage at room temperature (24 hours) in 0.1 n HCl, which produces 90 to 100 per cent loss for L. citrovorum, resulted in 41 to 48 per cent loss of SLR activity. Storage of FA under similar conditions results in no loss of activity for SLR. The implications of these results are considered in the discussion.

Sauberlich (5) has shown that rats fed FA excrete increased amounts of citrovorum-active material in urine. This suggests the in vivo conversion of FA to citrovorum factor. In limited trials in humans, 25 mg. of FA taken orally led to the excretion of but 2000 to 3000 citrovorum units in 24 hours. Assuming that the conversion of FA to the citrovorum factor results in no substantial change in molecular weight, from the tentative maximal activity ratio of SLR to citrovorum of 5, the 3000 citrovorum

^{*} SLR activity reported in terms of FA.

units are equivalent to only 0.015 mg. Thus if the *citrovorum* factor is a conversion product of FA, less than 0.1 per cent of the ingested dose was recovered in the urine in such form.

To check further on a possible relationship of the *citrovorum* factor to FA, a comparison was made of the rate of liberation of the two activities during the autolysis of normal rat liver. A fresh rat liver was minced in a Waring blendor and autolyzed at 37°. Samples were taken at the intervals indicated in Table III. The data show that the two activities are

Table III

Liberation of SLR and L. citrovorum Activity from Rat Liver

Digested	SLR*	Citrovorum	SLR
hrs.	mµgm. per gm.	units per gm.	
0	835	5.0	167
2	2237	52.0	43
4	2220	58.5	38
21.5	2337	149.0	16
24	2160	162.0	13

A 17 gm. rat liver was minced in a Waring blendor for 1½ minutes at 0° in 0.03 m phosphate-citrate buffer at pH 7.0. The suspension, under toluene, was then incubated at 37°.

liberated at different rates and that the *citrovorum*-active material appears to be released independently from that for SLR.

DISCUSSION

The data concerning the parallelism of SLR and L. citrovorum activity (Table II) are open to several interpretations. It seems likely at this time that the most highly purified concentrates contain two active substances, FA which is active for SLR only, and the citrovorum factor which is active also for SLR. On acid treatment of the citrovorum factor, activity for both organisms is lost. The residual SLR activity is due therefore to original contamination with FA.

On the other hand if the FA content of the concentrates is insignificant, then the *citrovorum* factor on acid degradation is converted to FA. The question as to which of these two hypotheses is correct must await the further purification of *citrovorum* factor.

In this connection it is of interest that Pfiffner et al. (6) have reported the occurrence in liver of an acid-labile material having FA activity for SLR. In view of the similar procedures employed in this concentration

^{*} Reported in terms of FA activity.

it seems likely that their acid-labile factor and the citrovorum factor are the same, or closely related.

SUMMARY

A method is described for the preparation of concentrates of the factor required for growth of *Leuconostoc citrovorum* 8081. Some of the properties of such concentrates are described. Preparations active for *L. citrovorum* were also active for *Streptococcus lactis* R.

The authors are indebted to Miss Rita Gardiner for many of the routine assays.

BIBLIOGRAPHY

- 1. Sauberlich, H. E., and Baumann, C. A., J. Biol. Chem., 176, 165 (1948).
- 2. Snell, E. E., Kitay, E., and McNutt, W. S., J. Biol. Chem., 175, 473 (1948).
- 3. Teply, L. J., and Elvehjem, C. A., J. Biol. Chem., 157, 303 (1945).
- 4. Lyman, C. N., and Prescott, J. M., J. Biol. Chem., 178, 523 (1949).
- 5. Sauberlich, H. E., Federation Proc., 8, 247 (1949).
- Pfiffner, J. J., Binkley, S. B., Bloom, E. S., and O'Dell, B. L., J. Am. Chem. Soc., 69, 1476 (1947).

THE PRELIMINARY CHARACTERIZATION OF TWO BIOTIN-CONTAINING FRACTIONS IN BEEF LIVER*

By KLAUS HOFMANN, DANIEL F. DICKEL, and A. E. AXELROD (From the Department of Chemistry, University of Pittsburgh, Pittsburgh)

(Received for publication, October 27, 1949)

A large fraction of the biotin in animal tissues has been assumed to be firmly bound to the tissues, and treatments known to destroy proteins such as acid hydrolysis or enzymatic digestion have been used routinely to liberate the vitamin from these materials for microbiological assay (1), Recently a study on "soluble bound forms" of biotin has been presented (2), and the isolation of a crystalline biotin complex (biocytin) active for Saccharomyces cerevisiae but not for Lactobacillus arabinosus has been reported (3). The present study was directed towards the isolation and characterization of biotin-protein complexes from animal tissues, since it seems likely that they represent the enzymatically active forms of biotin. Little information is available on the extractability from tissues of microbiologically inactive biotin-containing proteins. The presence of high molecular weight "saline-soluble" biotin complexes in egg yolk has been reported by György and Rose (4) who found that these materials although non-dialyzable had the same growth-promoting activity for yeast before and after hydrolysis with sulfuric acid.

Beef liver was selected as the starting material for the present investigation, since it is well established that this tissue contains biotin in a form which prior to hydrolysis is not available to microorganisms. The liver was dehydrated with acetone and the resulting acetone liver powder extracted under mild conditions. The resulting extracts were assayed by the yeast growth method for their biotin content before and after hydrolysis with acid. By the use of this technique it was found that beef liver contains at least two distinct biotin-containing fractions. One of these is extractable with a buffer at pH 3 and loses its biotin activity on dialysis; the other is soluble in dilute sodium hydroxide and is not affected by dialysis. The substance soluble at pH 3 has practically the same biotin activity before and after acid hydrolysis, in contrast to the alkali-soluble compound which has very little activity initially but becomes highly active following hydrolysis with sulfuric acid.

^{*} Supported in part by grants from Ciba Pharmaceutical Products, Inc., and the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

EXPERIMENTAL

Preparation of Liver Powder—Fresh beef liver was obtained from a slaughter-house and immediately cooled with ice. The liver was ground in small portions in a Waring blendor with ice-cold acetone and the slurry filtered with suction on a Büchner funnel. The resulting material was reground with acetone, filtered, dried at room temperature for 2 to 3 hours, and finally stored in the refrigerator. From 500 gm. of fresh liver, 140 gm. of liver powder were obtained. Samples of the liver powder were autoclaved with 6 N sulfuric acid for 1 hour at 18 pounds pressure for total biotin determination. The biotin content of several preparations ranged from 3.3 to 4.5 γ per gm. (S. cerevisiae assay).

Preparation of Extracts—The liver powder was extracted with distilled water, universal buffer solutions (5) (ranging from pH¹ 3.0 to 11.0), and 0.025 N sodium hydroxide. 2 gm. samples of the liver powder were weighed into 50 ml. centrifuge tubes and each sample extracted three times with 25 ml. portions of the respective solvent by shaking overnight at 5° in a cold room. The resulting suspensions were separated by centrifugation and the extracts from each liver sample combined (approximately 75 ml.) and made up to 100 ml. with distilled water.

The dialyzed samples were obtained by dialyzing the above extracts in Visking sausage casing against running distilled water for 48 hours at 5°.

Small aliquots (1 to 2 ml.) of the extracts were autoclaved with 5 ml. of 6 N sulfuric acid for 1 hour at 18 pounds pressure for the preparation of the acid-hydrolyzed samples. The samples were adjusted to pH 4 with dilute sodium hydroxide and suitable dilutions used for assay.

Microbiological Assay Procedure-S. cerevisiae 139 was used as the test organism throughout this study. The assays were carried out according to the procedure of Snell, Eakin, and Williams (6) as modified by Hertz (7).

The assay flasks containing the culture medium were sterilized by autoclaving and cooled. Suitable dilutions of the extracts were then added and the flasks inoculated. The organisms were grown in 50 ml. Erlenmeyer flasks at 30°; growth was measured turbidimetrically by the use of a Klett-Summerson photoelectric colorimeter.

RESULTS AND DISCUSSION

The extraction of acetone liver powder with buffers, dilute sodium hydroxide, or water led to the results illustrated in Fig. 1. It may be noted that the growth-promoting activity of the resulting extracts decreased with increasing pH (Curve I). The activity of the corresponding acid-

¹ Sufficient 0.2 N sodium hydroxide was added to a solution which was 0.04 M with respect to phosphoric, acetic, and boric acids to give buffers of the desired pH.

autoclaved extracts (Curve II) between pH 3 and 7 was practically identical with that of the non-hydrolyzed samples, while the biological activity of the alkaline extracts was markedly increased by the acid treatment. This difference in activity was most pronounced with the sodium hydroxide extract, which prior to hydrolysis (point A) was practically inactive

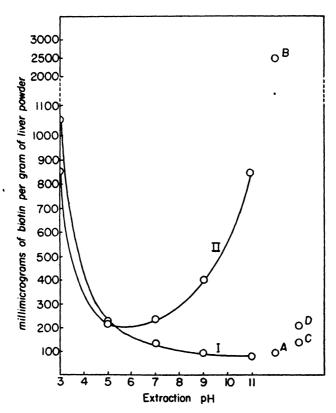


Fig. 1. Biotin activity of extracts obtained from acetone liver powder. Curve I, unhydrolyzed extracts; Curve II, hydrolyzed extracts. A, biotin content of NaOH extract before hydrolysis; B, biotin content of NaOH extract after hydrolysis; C, biotin content of water extract before hydrolysis; D, biotin content of water extract after hydrolysis.

but exhibited striking activity following autoclaving with acid (point B). It is also apparent that the material soluble at pH 3 was not extractable by dilute sodium hydroxide.

These results indicated that two different biotin-containing fractions were obtained from the liver powder, one with the pH 3 buffer and the other with sodium hydroxide. Neither fraction could be extracted with

distilled water (points C and D). Accordingly, experiments were undertaken with the aim of recovering both these materials from the same sample of liver powder by first extracting with universal buffer at pH 3 and then with dilute sodium hydroxide. The results of such an experiment are illustrated in Fig. 2, where it may be seen that both fractions

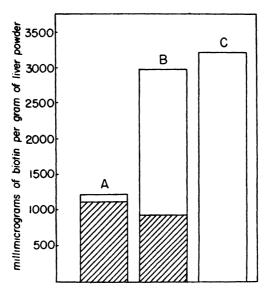


Fig. 2. Recovery of biotin in extracts from liver powder. A, biotin content of non-hydrolyzed extract at pH 3 (shaded) and non-hydrolyzed NaOH extract (clear). B, biotin content of hydrolyzed extract at pH 3 (shaded) and hydrolyzed NaOH extract (clear). C, total biotin content of liver powder.

Table I

Behavior of Liver Fractions on Dialysis
The figures represent millimicrograms of biotin.

Extract	Biotin bef	ore dialysis	Biotin after dialysis		
	Acid-hydrolyzed	Non-hydrolyzed	Acid-hydrolyzed	Non-hydrolyzed	
pH 3 NaOH		1000 220	80 1510	90 7 0	

could be recovered by this procedure. Again the pH 3 extract did not show an increase in biological activity following acid hydrolysis, in contrast to the alkali-soluble material which became highly active under these conditions. It is especially noteworthy that 90 to 100 per cent of the biotin present in the liver powder may be recovered in the two ex-

tracts. Attempts to recover the material soluble at pH 3 from liver powder previously extracted with sodium hydroxide were unsuccessful.

The different nature of the two fractions was further apparent from their behavior on dialysis as shown in Table I. It may be noted that dialysis for 48 hours resulted in an almost complete loss of the biological activity of the pH 3 extract. The alkali-soluble material is virtually non-dialyzable; its low activity prior to acid hydrolysis seems to be due to contamination with a dialyzable biotin-active compound of low molecular weight.

The lack of activity of the alkali-soluble material may be explained in two ways. Either the biotin is combined with a high molecular substance (protein) and is thus not available to the microorganism, or the extracts contain acid-labile inhibitory compounds which suppress its

Biotin content of extract	Biotin added	Recovery of added biotin		
mµgm.	mμgm.	mµgm.	per cont	
0.04*	0.02	0.02	100	
0.04*	0.05	0.04	80	
0.04*	0.15	0.14	93	
0.04*	0.20	0.23	115	
0.05†	0.02	0.03	150	
0.05†	0.05	0.05	100	
0.05†	0.10	0.12	120	

TABLE II

Recovery of Biotin Added to Sodium Hydroxide Extract

growth-promoting activity. The latter possibility was ruled out by recovery experiments summarized in Table II, in which known amounts of biotin were added to the non-hydrolyzed sodium hydroxide extracts. The excellent recoveries of biotin in the presence of either the dialyzed or non-dialyzed extract demonstrate the absence of inhibitory substances.

The existing nomenclature of the so called "bound forms" of biotin is at present in a confused state. Bowden and Peterson (2), for example, applied the term "soluble bound forms" of biotin to substances obtained by digestion of liver with pepsin or partial hydrolysis with sulfuric acid. Our findings which demonstrate that the biotin activity present in beef liver is extractable under mild conditions not involving such hydrolytic treatments suggest the need for a different nomenclature. In accordance with the accepted term flavoprotein, we propose that the term biotoprotein be applied to biotin-containing proteins as they occur in tissues. The

^{*} Non-hydrolyzed sodium hydroxide extract.

[†] Non-hydrolyzed dialyzed sodium hydroxide extract.

biotin activity may be available to microorganisms either before or after hydrolysis. We assume that our sodium hydroxide-soluble material represents such a biotoprotein. The low molecular weight biotin-containing portion of this biotoprotein may well be identical with the substance described by Bowden and Peterson (2). Experiments to test this hypothesis are presently under way. The similarity in the bacterial spectrum of biocytin and Bowden and Peterson's material indicates the possible identity of these two compounds.

The nature of the material soluble at pH 3 is less clearly definable, as it contains biotin activity in a rather loose combination which is cleaved by simple dialysis against distilled water. The solubility characteristics make it rather unlikely that this material is biotin.

Recently it has been suggested that biotin may in some manner function in the fixation of carbon dioxide (8-12). Several enzymes involved in the decarboxylation of β -keto acids have been obtained in purified form; however, in no instance could the enzymatic activity be correlated with the biotin content (10, 13, 14). Thus it becomes evident that the elucidation of the mode of action of biotin will depend in the final analysis on the isolation in pure form of biotin-containing enzymes and the identification of their specific catalytic activity. The work herein described represents a first step towards this goal.

SUMMARY

The biotin activity present in acetone-dried beef liver was almost quantitatively extracted by successive treatments with universal buffer at pH 3 and dilute sodium hydroxide. Two distinct fractions containing biotin were thus obtained. The substance soluble at pH 3 possessed biotin activity which was not increased by acid hydrolysis; on the other hand, the sodium hydroxide-soluble material exhibited activity only after treatment with acid. In contrast to the fraction soluble at pH 3 which lost its biotin activity on dialysis, the biological activity of the alkali-soluble substance remained practically unaltered by this treatment. The chemical nature of the alkali-soluble substance was discussed and it was postulated to be a biotin-protein complex. The name biotoprotein was proposed for native biotin-containing proteins occurring in tissues.

The technical assistance of Mrs. Donna Kaiser is gratefully acknowledged.

BIBLIOGRAPHY

- 1. Hofmann, K., Advances in Enzymol., 3, 289 (1943).
- 2. Bowden, J. W., and Peterson, W. H., J. Biol. Chem., 178, 533 (1949).

- Wright, L., Gordon Research Conferences, American Association for the Advancement of Science, on vitamins and metabolism, Aug. (1949).
- 4. Gyorgy, P., and Rose, C. S., Proc. Soc. Exp. Biol. and Med., 49, 294 (1942).
- 5. Prideaux, E. B. R., Proc. Roy. Soc. London, Series A, 92, 463 (1916).
- 6. Snell, E. E., Eakin, R. E., and Williams, R. J., J. Am. Chem. Soc., 62, 175 (1940).
- 7. Hertz, R., Proc. Soc. Exp. Biol. and Med., 52, 15 (1943).
- 8. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., J. Biol. Chem., 169, 451 (1947).
- 9. Lichstein, H. C., and Umbreit, W. W., J. Biol. Chem., 170, 329 (1947).
- Ochoa, S., Mehler, A., Blanchard, M. L., Jukes, T. H., Hoffmann, C. E., and Regan, M., J. Biol. Chem., 170, 413 (1947).
- 11. Lardy, H. A., Potter, R. L., and Burris, R. H., J. Biol. Chem., 179, 721 (1949).
- 12. MacLeod, P. R., and Lardy, H. A., J. Biol. Chem., 179, 733 (1949).
- 13. Vennesland, B., Gollub, M. C., and Speck, J. F., J. Biol. Chem., 178, 301 (1949).
- 14. Plaut, G. W. E., and Lardy, H. A., J. Biol. Chem., 180, 13 (1949).

A MODIFICATION OF THE CARBAZOLE REACTION OF HEXURONIC ACIDS FOR THE STUDY OF POLYURONIDES*

By ZACHARIAS DISCHE

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, September 10, 1949)

In a previous report a specific color reaction of hexuronic acids with carbazole and sulfuric acid was described (1). In this reaction glucuronic and galacturonic acids, in free as well as in combined form in polyuronides, reacted with almost equal intensity. By decreasing the concentration of the acid and the temperature and time of heating, a modification of the carbazole reaction was obtained in which the intensity of the color depends not only on the structure of individual hexuronic acids but also on specific linkages in polyuronides. The present report deals with this modification. It will be referred to as the reaction at 60° to distinguish it from the first modification, the reaction at 100°.

EXPERIMENTAL

Procedure—5.4 cc. of a mixture of 1 part of water plus 6 parts of concentrated sulfuric acid, c.p., are pipetted into test-tubes of equal inner and outer diameter, which are immersed in ice. The acid is cooled to 0°, 0.4 cc. of a solution containing 0.05 to 0.5 mg. of hexuronic acid per cc. is added, and the mixture is shaken in ice. After the reaction mixture has cooled to 0°, it is held for 3 minutes in tap water at room temperature and immersed immediately in a water bath at 60° for 90 seconds. It is then cooled in tap water, and 0.2 cc. of a 0.1 per cent solution of carbazole (Eastman Kodak preparation, twice recrystallized from benzene) is added and vigorously shaken. A purple color develops slowly and increases in intensity for several days. To obtain as great differences in color intensity as possible with individual hexuronic acids and polyuronides, the intensity of the color should be measured 1 hour after addition of carbazole against a blank containing 0.4 cc. of water instead of the unknown solution. a Klett photoelectric colorimeter is used as the optical instrument, the maximum absorption is obtained with Filter 54. With the Beckman spectrophotometer, the maximum absorption is found at 527 mµ.

Sensitivity and Specificity of Reaction-The color can be detected with

^{*} This work was supported by a grant of the Donner Foundation, Inc., Cancer Research Division.

solutions containing as little as 0.05 mg. per cc. of galacturonic or 1 mg. per cc. of glucuronic acid. The reading on the Klett colorimeter in this case is about 20. Hexoses, pentoses, methylpentoses, and desoxyribose nucleic acid do not give an appreciable color even in concentrations of 0.5 mg. per cc.

Influence of Proteins—The intensity of the color reaction is influenced by the proteins in the solution. Serum proteins at a concentration of 0.1 per cent depress the intensity of the color by 25 per cent. The effect is not perceptible with 0.02 per cent solutions. Therefore, if the concentration of hexuronic acids is not high enough to allow a dilution sufficient to decrease the protein concentration below 0.02 per cent, it is necessary to determine the percental depressing effect of the protein on the color reaction. This can be done by adding a known amount of galacturonic acid to a certain volume of solution and measuring the increase of color against a standard prepared by adding the same amount of hexuronic acid to a corresponding volume of water.

Intensity of Color Given by Various Hexuronic Acids and Polyuronides in Reaction

The intensity of color given in this procedure by equivalent amounts of hexuronic acids in free and conjugated form shows (Table I) great variations with the nature of the acid and of the polyuronide. Free galacturonic acid yields a color 30 times more intense than that from glucuronic acid. Among the polyuronides, two groups with very different behaviors are conspicuous. To the first group belong the two pneumococcus polysaccharides (types I and III) and pectic acid. Their reaction is negligible. They behave in the same way as the β -mentholglucuronide. It is obvious that the glycosidic linkage by which the hexuronic acid is bound in these compounds depresses the color they would show as free hexuronic acids.

The second group is represented by the three mucopolysaccharides containing an amino sugar: the hyaluronic and chondroitinsulfuric acids from cartilage, heparin, and alginic acid. In spite of the decrease in color due to glycosidic linkages, these polyuronides show a considerable intensity of color which, in the case of hyaluronic acid, is about 20 times stronger than that from an equivalent amount of the free glucuronic acid¹ (measured with Filter 54 in the Klett photoelectric colorimeter).

Extinction Coefficient in Color Reaction as Characteristic of Polyuronides

The great variations in the reactivity of different polyuronides in this reaction permit their characterization by the extinction coefficient, ϵ_{527} .

¹ The ratio between color intensities of glucuronic acid and polyglucuronides cannot be determined very accurately because of the low values obtained for glucuronic acid. This ratio depends also somewhat on the room temperature.

After the total amount of hexuronic acid in a preparation or unknown solution containing polyuronides is determined (by one of the modifications of the Lefèvre-Tollens method), the intensity of color as determined in the

Table I

Color Intensity Given by Various Hexuronic Acids and Polyuronides in

Reaction at 60°

Readings 1 hour after addition of carbazole.

Experiment No.	Substance	Concen- tration hexuronic acid	Galvanom eter reading*
		mg. per cent	
I	Glucuronic acid	83.3	18
	Chondroitinsulfuric acid†	85	62
	Chondridin	100	83
11	Glucuronic acid	83.3	13
	Chondroitinsulfuric acid	85	53
	Hyaluronic acid	168	250
III	Pneumococcus type III polysaccharide	90	3
IV	Glucuronic acid	80	14
	β -Menthoglucuronide	80	0
	Hyaluronic acid from corpus vitreum	80	189
	Hyaluronic acid from umbilical cord	80	210
	Chondroitinsulfuric acid	80	52
	Galacturonic acid	25	84
1	Pectic acid	25	8
V	Galacturonic acid	12.5	46
	Pectic acid	12.5	0
	β -Mentholglucuronide	100	0
	Hyaluronic acid from corpus vitreum	40	70
VI	Glucuronic acid	50	3
	Pneumococcus type I polysaccharide	100	7
	Chondroitinsulfuric acid	40	16
VII	Glucuronic acid	100	10
	Hyaluronic "from umbilical cord	100	214
1	Galacturonic acid	25	75
1	Pectic acid	25	5

^{*} Readings made with a Klett photoelectric colorimeter.

present method will enable one in many cases to detect the nature of the polyuronide. To this end, the density of color formed from our preparation is compared with that given by a standard solution of glucuronic or galacturonic acid containing as much total hexuronic acid as the unknown.

[†] All preparations of chondroitinsulfuric acid used in this investigation were prepared from cartilage. I am greatly indebted to Dr. Karl Meyer for the preparations of hyaluronic and chondroitinsulfuric acids and to Dr. Michael Heidelberger for the bacterial polysaccharides.

The relative density of the two solutions will indicate the nature of the polyuronide.

The determination of the total hexuronic acid, according to Lefèvre and Tollens, can be replaced by the determination of the intensity of color given by the unknown solution in the carbazole reaction at 100° previously described (1). To this end, the relative density of the unknown is determined in the two procedures by comparison with standard solutions containing 0.05 to 0.1 mg. of galacturonic acid per cc. The relative density

Table II

Ratio of Relative Densities of Reactions at 60 and 100° $D_{\rm Re0^\circ}/D_{\rm R100^\circ}$ for various polyuronides against a standard of galacturonic acid of 25 mg. per cent.

Experi- ment No.	Tem- pera- ture	Substance	Concentration hexuronic acid	Galvanom- eter reading*	D _{Res} °	D _{R100} *	D _{Reso} °
	°C.		mg. per cent				
I	24	Hyaluronic acid from corpus vitreum	100	214	0.72	1.42	0.51
		Pectic acid	25	5	0.06	1	0.06
İ		Galacturonic acid	25	75			
11	28	Hyaluronic acid from corpus vitreum	80	189	0.76	1.42	0.53
		Chondroitinsulfuric acid from cartilage	80	52	0.19	1.14	0.17
		Galacturonic acid	25	84			
III	30	Pneumococcus type I poly- saccharide	12.5	0	0	1.15	
ļ		Alginic acid	40	61	0.51	0.25	2
}		Galacturonic acid	25	74			
IV	28	Heparin crystalline Ba salt	20	74	1.21	2.05	0.59
		Galacturonic acid	25	76			

^{*} Readings made with a Klett photoelectric colorimeter.

obtained in the reaction, which involves heating at 100°, of equimolar solutions of all hexuronic acids and polyuronides so far investigated is very nearly the same. The ratio of the relative densities given by the two methods designated $D_{\rm R00^{\circ}}/D_{\rm R100^{\circ}}$, therefore, is as characteristic for many polyuronides (hyaluronic, chondroitinsulfuric, and pectic acids) as the extinction coefficient in the reaction at 60° (see Table II).

DISCUSSION

The new modification of the carbazole reaction promises to be useful for three different lines of investigation. z. dische 493

Preliminary experiments have shown that the intensity of the color formed following the reaction at 60° is proportional to the concentration of galacturonic acid in the range between 0.05 to 1.0 mg. per cc. and of hyaluronic acid and heparin between 0.05 and 0.25 mg. per cc. Therefore, it should be possible to determine these compounds in the presence of an excess of free glucuronic acid, β -glucuronides (in urine), and other polyuronides.

The 60° and 100° reactions can be used in combination for testing the purity of preparations of polyuronides, at least as far as contamination by other polyuronides is concerned. To this end, the ratio $D_{\rm R00^{\circ}}/D_{\rm R100^{\circ}}$ is compared with the ratio of a reference preparation of known purity. Any impurity in the form of a polyuronide should, in general, give rise to a difference in this ratio in the two preparations.

Finally, the close relation between the intensity of color and certain structural peculiarities of polyuronides should make it possible to differentiate between and to characterize certain closely related mucopolysaccharides isolated from different animal tissues. Meyer et al. (2) have reported that preparations of chondroitinsulfuric and mucoitinsulfuric acids from various organs show considerable differences as far as certain physicochemical properties (viscosity, rotation) and sensitivity towards enzymes are concerned. These authors considered the possibility that these differences in behavior indicate differences in structure. These physicochemical properties and the susceptibility to enzyme activity, however, cannot be regarded as unequivocal criteria of the chemical individuality of compounds of the character of mucopolysaccharides. The degree of polymerization of the latter, as well as impurities, may affect those properties. On the other hand, experiments² have shown that the intensity of color given by hyaluronic acid in the reaction at 60° does not depend on the degree of polymerization. Normal sulfuric acid, which at 20° strongly affects the viscosity of solutions of hyaluronic acid, does not affect its chromogenic properties at all. Obviously this reaction depends on a specific structure of a low molecular unit of the polysaccharide.

The fact that certain polyuronides like hyaluronic acid yield much more color following the reaction conducted at 60° than does free glucuronic acid can be easily understood if one considers the general mechanism of the breakdown of sugars in strong acids which precedes the color reactions with the organic chromogenic reagent. As a rule, several intermediary products more or less related to, or derived from, furfural and its homologues will arise. Of these, in general, only one will combine with the developer to form the colored product ("active intermediary"). The ratio between the amount of the active and the other intermediaries will often

² Unpublished data.

depend on the presence of free hydroxyl groups in certain positions in the sugar molecule. If one of these groups is bound by a glycosidic linkage, a shift of this ratio towards the active intermediary may result. If the active product is present in much lower concentration than the inactive (as was shown to be the case in the diphenylamine reaction of desoxypentose (3)), such a shift may result in a considerable increase in the intensity of the color reaction.

SUMMARY

A modification of the carbazole reaction for hexuronic acids is described, which permits a differentiation between galacturonic and glucuronic acids and between various types of polyuronides.

BIBLIOGRAPHY

- 1. Dische, Z., J. Biol. Chem., 167, 189 (1947).
- 2. Meyer, K., and Chaffee, E., J. Biol. Chem., 138, 491 (1941).
- 3. Stacey, M., Deriaz, R. E., Teece, E. G., and Wiggins, L. F., Nature, 157, 740 (1946).

A STUDY OF THE BASIS OF SELECTIVITY OF ACTION OF ANTIMETABOLITES WITH ANALOGUES OF PIMELIC ACID

By D. W. WOOLLEY*

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, October 4, 1949)

Since selectivity of action of a drug is one of the prime factors which determines its therapeutic usefulness, any attempt to approach chemotherapy of both infectious and non-infectious diseases by means other than those dependent on chance must necessarily require some insight into the reasons why pharmacological agents show the ability to affect some organisms or tissues, and not to touch others. In all probability, a variety of bases for this selectivity exists. This paper is concerned with the investigation of one of them.

The selectivity of sulfanilamide and its related drugs in being able to harm bacteria but not to destroy higher animals has been postulated (1) to arise from the fact that the animals cannot synthesize folic acid, while the pathogenic microorganisms can do so. These drugs are antimetabolites of p-aminobenzoic acid and apparently owe their effects to competition with this metabolite in the synthesis of folic acid (2). This idea about the reason for their selective action arose from the observation of Lampen and Jones (3) that those bacteria which require folic acid preformed are not harmed by the drugs. Because most higher animals require dietary folic acid, they lack the metabolic system which sulfonamide drugs inhibit, and therefore are not vitally affected by them, while pathogenic bacteria which do form folic acid may be susceptible to the inhibition.

In order to test this postulate further, it was applied to another vitamin in an effort to make a selective agent. An antimetabolite related to the precursor of the vitamin was therefore prepared and tested on a series of living things. Biotin was selected for this trial because pimelic acid has been shown to be a specific precursor from which, by a series of reactions, the vitamin is formed (4). Furthermore, many animal species seem to require a dietary source of biotin, while some pathogenic microorganisms, e.g. the tubercle bacillus, do not. If the hypothesis is correct, one would expect that an effective analogue of pimelic acid would harm those organisms which can synthesize their own biotin, while it should not affect those animals and microorganisms which do not. Furthermore, the harm-

^{*} With the technical assistance of N. Smith and J. Cowperthwaite.

ful effects of such an analogue should be overcome competitively by pimelic acid and non-competitively by small amounts of biotin.

Analogues of pimelic acid which will do these things in the species thus far examined have been made. The most active one was the ϵ -dichlorosulfanilide of caproic acid. Its structure and that of pimelic acid are shown in the diagram. The simpler analogue in which a plain sulfonamido group replaced the dichlorosulfanilido radical was effective, but with the bacteria tested the inhibition of growth which it caused disappeared as the time of incubation was increased. However, the monosulfonic acid analogue of pimelic acid, namely ϵ -sulfonocaproic acid, was not harmful to any organism tested, and therefore was not an antimetabolite of pimelic acid.

COOH

(CH₂)₅
COOH

Pimelic acid

$$E-[2,4-Dichloro-sulfanilido]$$
- caproic acid

The successful prediction of these results by use of the hypothesis lends some support to it as an explanation of one of the bases of selectivity of action of antimetabolites. It does not constitute proof of its validity but does make it seem advisable to explore it further.

EXPERIMENTAL

Sodium and Barium Salts of ϵ -Sulfonoethyl Caproate—To a hot solution of 22 gm. of ϵ -bromoethyl caproate (5) in 50 cc. of ethanol was added slowly with stirring a solution of 23 gm. of Na₂SO₃ in 40 cc. of water. The mixture was heated on the steam bath under a reflux and stirred vigorously for 1 hour.¹ It was then concentrated under reduced pressure to dryness and the residue was extracted with 400 cc. of hot ethanol. After filtration the solution was concentrated under reduced pressure to a sirup which was extracted with 400 cc. of ether. This removed 7.7 gm. of unchanged bromo ester. The ether-insoluble matter was dissolved in ethanol and precipitated by the addition of 10 volumes of ether. This operation

¹ This time of reaction was chosen arbitrarily. Systematic investigation might reveal conditions which would minimize hydrolysis of the ester and thus increase the yield.

was repeated twice; yield 12 gm. of white powder. This material was mainly the sodium salt of ε-sulfonoethyl caproate but contained some of the disodium salt of the corresponding caproic acid. The separation of these two sodium salts proved difficult, but the two acids could be parted completely as barium salts. To do this 500 mg. of the mixed sodium salts were dissolved in 25 cc. of ethanol and a solution of 500 mg. of barium iodide in 10 cc. of ethanol was added. Shiny crystals of the barium salt of ε-sulfonoethyl caproate soon began to form, and these were recrystallized by adding 4 volumes of ethanol to a 10 per cent solution of them in water. For the preparation of the sulfonamido and sulfanilido compounds, the mixed sodium salts were satisfactory.

C₁₆H₈₀O₁₀S₂Ba. Calculated. C 33.0, H 5.2, Ba 23.6 Found. " 32.5, " 5.2, " 23.8

ε-Sulfonamidocaproic Acid—2.5 gm. of the sodium salt of ε-sulfonoethyl caproate (mixed with small amounts of the disodium salt of the corresponding caproic acid) were ground in a mortar with 5 gm. of PCl₅. When the reaction had subsided, the mixture was extracted with 50 cc. of dry benzene and the filtered solution was concentrated under reduced pressure to dryness. Dry benzene was added and the evaporation was repeated to remove HCl and POCl₃. The acid chloride crystallized but was used without further purification. 100 cc. of concentrated aqueous ammonium hydroxide were added and the solution which resulted was kept at room temperature for 2 days, and then concentrated under reduced pressure to dryness. The diamide of ϵ -sulfonocaproic acid was separated by extraction of the residue with absolute ethanol and fractional precipitation with ethyl acetate. It did not crystallize, but rather formed as an amorphous powder. This was therefore dissolved in 25 cc. of 6 N HCl and the solution was refluxed for 4 hours. The reaction mixture was concentrated under reduced pressure to dryness, dissolved in water, and from this solution the sulfonamide was extracted with ethyl acetate. Evaporation of the extract left a crystalline substance which was recrystallized from ethyl acetate solution by adding benzene. Yield 660 mg.; m.p. 102-104°.

C₆H₁₃O₄NS. Calculated, N 7.2; found, N 7.3

 ϵ -(2,4-Dichlorosulfanilido)-caproic Acid—The acid chloride from 2.5 gm. of the crude sodium salt of ϵ -sulfonoethyl caproate was prepared as described in the previous section and was dissolved in 10 cc. of dry benzene. This solution was added to 4 gm. of 2,4-dichloroaniline dissolved in 20 cc. of benzene. Since reaction seemed slow the solvent was removed under reduced pressure and the residue was heated at 120° for 30 minutes. The product was dissolved in ethyl acetate, and this solution was washed sev-

eral times with 1 N HCl and then with water. The crude sulfanilido ester which was obtained by concentration of the ethyl acetate was not purified further, but rather was converted to the free acid by refluxing it for 2 hours in 32 cc. of 1 N alcoholic NaOH. The ethanol was removed under reduced pressure, the residue was dissolved in water, and the solution was extracted with ethyl acetate. Acidification of the aqueous phase gave 1.5 gm. of the desired acid which was recrystallized by adding benzene to an ethyl acetate solution. It melted at 115–118°.

C₁₂H₁₅O₄NSCl₂. Calculated, C 42.4, H 4.41; found, C 42.4, H 4.65

Method of Testing with Microorganisms—To test a pimelic acid analogue for ability to inhibit growth of microorganisms, the substance was added to a medium free of biotin and of pimelic acid. Graded amounts ranging from 1 mg. per cc. of culture medium down to 1 γ were so tested with each species and with each compound. For those forms which needed biotin for growth, an amount of this vitamin equal to about half that which gave maximal response was included in the basal medium. For all except Acetobacter, Saccharomyces, and the tubercle bacillus, the basal medium described by Landy and Dicken (6) was used. In order to minimize any possible interference with the inhibitory action of the analogues due to excess amino acids, the amount of casein hydrolysate in the basal medium was reduced to one-fourth that recommended. With this quantity of amino acids, excellent growth was still obtained. The basal medium therefore contained glucose, acid hydrolysate of casein, tryptophan, cystine. asparagine, sodium acetate, potassium phosphate, sodium chloride, salts of iron, manganese, and magnesium, adenine, guanine, xanthine, uracil, thiamine, riboflavin, pantothenic acid, pyridoxine, pyridoxamine, nicotinic acid, folic acid, and p-aminobenzoic acid. Acetobacter suboxydans was tested in the medium described for it by Landy and Dicken (7), and for Saccharomuces cerevisiae the synthetic medium used by Woollev and White (8) was employed. The experiments with Mycobacterium tuberculosis were conducted by Dr. R. J. Dubos in the basal medium which he has developed for this organism (9).

When one of the analogues was found to inhibit the growth of a given species, additional tests were made to determine (a) whether pimelic acid would overcome the inhibition, (b) whether biotin would overcome the inhibition, and (c) whether these antagonisms were competitive. To do this, a series of graded dilutions of the analogue was tested in the presence of no added metabolite, with 1γ per cc. of pimelic acid, with 3γ per cc. of pimelic acid, and with 0.0005γ and with 0.005γ of biotin. For each amount of either metabolite the quantity of the analogue which gave half maximal inhibition of growth was determined from a plot of the dose-

response curve. Inspection of these values then readily showed whether antagonism existed, and if so, what its nature was. A competitive antagonism would demand a 3-fold increase in analogue when 3 times as much metabolite was present, in order to achieve similar effect. If it were non-competitive, the same amount of analogue would suffice at all levels of the metabolite. Except where noted otherwise, all periods of incubation were 2 days at 37°. Extent of growth was measured quantitatively with an Evelyn colorimeter. A. suboxydans, S. cerevisiae, Lactobacillus arabinosus, and Leuconostoc mesenteroides were grown at 30° instead of 37°. The organisms were all cultured in 25 × 100 mm. test-tubes containing 10 cc. of medium except in the case of A. suboxydans, S. cerevisiae, and Bacillus tenuis, in which cases 50 cc. Erlenmeyer flasks were used. This manner of cultivation was important, as will be shown below for B. tenuis.

Microorganisms Used—The selection of organisms for testing depended on their nutritional needs for biotin. While biotin-requiring species are common, those with no dependence on this vitamin are rare. Some forms which previously had been reported not to require biotin were found to do so provided that the inoculum was thoroughly washed and diluted. B. tenuis, a close relative of Bacillus subtilis, was found very useful in this work, because although it would grow without added biotin, addition of this vitamin enhanced multiplication. Furthermore, pimelic acid would replace biotin in this respect. A. suboxydans likewise grew somewhat without biotin and was stimulated when it was present, but pimelic acid was not able to replace it.

B. tenuis and Proteus 4 were kindly supplied by Dr. R. J. Dubos. A. suboxydans was from the American Type Culture Collection. A biotin-requiring mutant of Escherichia coli was kindly supplied by Dr. B. Davis and Dr. W. Maas. All other species were those previously in use in this laboratory.

Stimulation of Growth of B. tenuis by Pimelic Acid or by Biotin—When B. tenuis was inoculated into the biotin-free basal medium just described, growth was scant and slow to begin, unless either biotin or pimelic acid was present. This effect was regularly noted when 10 cc. of medium were employed in 25 × 100 mm. test-tubes. However, when the cultivation was in 50 cc. Erlenmeyer flasks, growth in the unsupplemented basal medium was faster, and the stimulation by biotin and by pimelic acid was not evident. In the trials in test-tubes, growth was at first diffuse throughout the medium. In the unsupplemented tubes, this diffuse character remained. In the supplemented tubes growth began as a uniform turbidity but a pellicle soon developed on the surface. These pellicles complicated the determination of the amount of growth because grinding was

representative experiment on beef hemolysate. It may be seen that, as expected, the beef cells are inactive in the formation of stable P. The moderate activity in liberating inorganic P, particularly in the complete

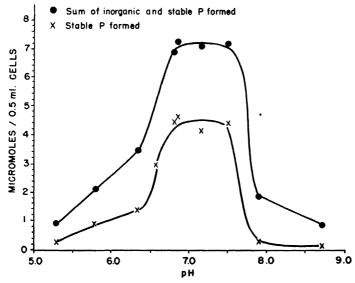


Fig. 4. pH-activity curve of rabbit hemolysate. The experimental conditions were the same as those described in Table I. Different pH values were produced by additions of HCl or NaOH of appropriate strength, and the exact values determined with a glass electrode.

TABLE II

Effect of Dilution of Hemolysate

The conditions were the same as those described for Experiment A in Table I, except for the variation in the amount of red blood cells added, as indicated in the table. The values are expressed in micromoles.

Red cells per ml.	Inorganic P formed		Stable P formed		
mixture	Actual	Per 0.5 ml. cell	Actual	Per 0.5 ml. cell	
ml.					
0.167	1.7	5.1	1.4	4.3	
0.083	1.3	8.1	0.6	3.3	
0.042	0.7	8	0.1	1	
0.021	0.4	9		-1	

system containing both ATP and 3-P-GA, is an indication of the presence of the Bücher enzyme. In Table IV are shown data on experiments with acetone powder of a water extract of rat muscle. It may be seen that no

formation of stable P occurred; rather, an increase in the total hydrolyzable P took place. A comparison of the 150 minute P values of Tubes 4 and 6 shows that the increase was primarily due to a breakdown of 3-P-GA,

TABLE III Reef Cell Experiment

The experiment was set up in the same fashion as that described in Table I, except for the use of a 1:3 hemolysate of beef cells instead of rabbit cells. The samples were incubated for 4 hours at 37°. The values are expressed in micromoles per 0.5 ml. of red blood cells.

Experimental condition	Inorg	anic P	150 min. hydrolysis		
Daponinones conditions		Total	Δ	Total	Δ
Tube O + ATP + 3-P-GA " A + " + " " B + "	Initial Incubated	2.2 4.3 3.5	+2.1 +1.3	17.8 17.8 18.1	0.0 +0.3

TABLE IV Muscle Experiment

The experiment was performed as described in Table I, except that the mixtures contained approximately 4 μ M per ml. of ATP. 0.1 ml. of a 10 per cent suspension of acetone powder of water extract of rat muscle, adjusted to pH 6.8 with NaHCO₁, was added per ml. of final mixture. The values are expressed as micromoles per 0.1 ml. of muscle suspension.

Experimental conditions			Inor	Inorganic P		150 min. hydrol- ysis	
			Total	Δ*	Total	Δ*	
Tube 1 + muscle alone		Initial	3.1		3.6		
"2+""		Incubated	3.5	+0.4	3.8	+0.2	
" $3 + \text{muscle} + \text{ATP}$	+ 3-P-GA	Initial	4.7		12.7		
" 4 + muscle + ATP	44	Incubated	7.3	+2.6	14.0	+1.3	
" $5 + \text{muscle} + \text{ATP}$		"	5.0	+0.3	12.5	-0.2	
" 6 + muscle	+ 3-P-GA	"	4.4	+0.9	4.9	+1.1	

^{*} Differences of Tube 2 versus Tubes 1 and 4, Tube 5 versus 3, and Tube 6 versus 2.

presumably owing to an incomplete inhibition of enclase. The presence of an active Bücher enzyme is indicated by the high inorganic P value in Tube 4, which contained both ATP and 3-P-GA.

Effect of Muscle on Erythrocytes-It appeared of interest to study the

effect of the muscle preparation on the formation of stable P in hemolysates. An accelerating effect of the muscle, by itself inactive but containing a potent Bücher enzyme, would strongly support the hypothesis of a two-step reaction. In Table V are presented two such experiments, one at 37° and the other at 25°, both of which show a significant increase

TABLE V

Accelerating Effect of Muscle on Formation of Stable P in Rabbit Hemolysate

The conditions were the same as those described previously, except that the mixtures contained approximately 4 μ m per ml. of ATP. 0.1 ml. of a 10 per cent suspension of acetone powder of water extract of rat muscle, adjusted to pH 6.9 with NaHCO₂, was added per ml. of mixture. In one experiment unneutralized muscle suspension, pH 6.2, was used. The incubation times and temperatures are given in the table. The values are expressed as micromoles per 0.5 ml. of red cells.

Experim	ental conditions	Tube	Inorganic P formed	Stable P formed	
hrs.	°C.				
4	25	With muscle	7.2	2.5	
•	1	Without muscle	3.4	1.7	
2	37	With muscle, pH 6.2	5.5	3.7	
		"""6.9	6.3	2.3	
	1	Without muscle, pH 6.8	2.6	1.8	

TABLE VI

Effect of Increasing Amounts of Muscle on Stable P Formation

The conditions were the same as in Table V. Varying amounts of a 10 per cent aqueous suspension of acetone powder of muscle extract, adjusted to pH 6.5, were added. The samples were incubated for 4 hours at 37°. The values are expressed as micromoles per 0.5 ml. of red cells.

Muscle suspension per ml. final volume	per ml. final volume Inorganic P formed	
ml.		
0.0	3.8	4.3
0.05	5.7	4.5
0.10	7.7	6.9
0.15	8.7	7.0
0.20	9.7	7.0
0.10 (No 3-P-GA)	6.7	0.0

in the formation of stable P when the muscle powder was added. The effect of varying concentrations of muscle powder on the red blood cell system is presented in Table VI. It may be seen that, although the inorganic P formation increased with increasing amounts of muscle, a plateau of stable P formation was reached. In other experiments, even inhibition occurred with the largest additions of muscle powder.

Fractionation of Rabbit Hemolysate with Ammonium Sulfate—More decisive evidence concerning the two-step mechanism of 2,3-P-GA formation could be obtained by fractional precipitation of the hemolysate with ammonium sulfate. The results of an illustrative experiment are shown in

TABLE VII

Ammonium Sulfate Fractionation of Rabbit Hemolysate

About 20 ml. of rabbit red blood cells, washed twice with cold 0.9 per cent NaCl solution, were hemolyzed by freezing and by addition of 1 volume of distilled water. To the hemolysate which had a pH of 6.9, 0.33 volume of saturated ammonium sulfate solution was added to produce 0.25 saturation. The precipitate containing most of the stroma was separated by centrifugation, and to the clear red supernatant fluid were added, in succession, amounts of saturated ammonium sulfate solution sufficient to produce the saturations listed in the table. The bulk of the hemoglobin was contained in the 0.75 fraction. The precipitates were taken up in small volumes of distilled water and dialyzed overnight against running distilled water. The precipitates which appeared in the dialyzing bags were removed since they proved inactive. The combined fractions 0.50 and 0.66, which contained most of the activity and little hemoglobin, were brought to a pH of 5.8 by addition of 0.1 volume of 0.2 m acetate buffer and refractionated with ammonium sulfate. The various precipitates were redissolved in distilled water and dialyzed. They were practically free of hemoglobin.

Activity tests were performed in a manner similar to that described in Table V with and without addition of acetone powder of muscle. The inorganic P values for the tests with muscle are omitted from the table since they offer no information beyond indicating activity of the muscle powder. The values are expressed as micromoles of P per total fraction. The initial total activity of the red cells was about 180 μ M of stable P formed in 4 hours.

Fraction No. pH	ы	Ammonium sulfate	Inorganic P	Stable P formed		
	saturation	formed	Without muscle	With muscle		
I	6.9	0.25	57	9	55	
11		0.50	29	23	55	
111		0.66	20	24	62	
IV		0.75	10	3	3	
III + III						
:L	5.8	0.33	7	0	0	
b		0.50	13	0	35	
c		0.66	8	0	29	
d		0.80	6	0	16	

Table VII. It may be seen that the enzyme tended to distribute itself in the fractions precipitated by 0.50 to 0.75 saturation with ammonium sulfate. A single fractionation reduced greatly the amount of stable P formed without addition of muscle enzyme. On repeated fractionation of Fractions II and III, 0.50 and 0.66 saturated with ammonium sulfate, stable P formation no longer was demonstrable without addition of muscle

powder. Liberation of inorganic P decreased to a small per cent of the original value.

DISCUSSION

The data obtained leave little doubt that formation of 2,3-diphosphoglycerate was observed under circumstances which establish ATP and 3-P-GA as precursors. The evidence concerning a two-step reaction, the first catalyzed by the Bucher enzyme leading to production of 1,3-P-GA which then is transformed to 2,3-P-GA by a mutase, consists in the retarding effect of dilution on the synthesis, the accelerating effect of muscle powder, and in the fractionation of the hemolysate with ammonium sulfate until formation of stable P no longer occurred in the absence of added muscle powder. The enzyme, which may be called "diphosphoglycerate mutase," presumably is restricted to erythrocytes containing 2,3-P-GA. From the data of Bücher (10) one may conclude that in the present experiments the concentration of 1,3-P-GA must have been very low, much lower than the preexistent 2,3-P-GA concentration in the hemolysates. Occurrence of synthesis under such conditions suggests that the equilibrium of the mutase reaction must be far to the side of the stable compound. Aside from this observed fact, one may raise the question on thermodynamic grounds as to whether the reaction can be reversible at all, in view of the great differences in energy content between the carboxyl phosphate of 1,3-P-GA on the one hand and that of the secondary alcohol phosphate of 2,3-P-GA on the other. An energy difference of 13,000 calories between the two types of groups may be estimated. Such an objection would overlook the fact that a similar system, namely that between 2-P-GA and phosphopyruvate, is freely reversible, with little change in energy, and has an equilibrium constant of 2 to 3 (12). This apparent paradox is explained by the fact that the energy change at the phosphate bond is compensated by an equivalent change in the phosphate-free portion of the compound. Thus free glyceric acid is richer in energy than either pyruvic acid or the acid anhydride (13). In view of these considerations the occurrence of a back-reaction cannot be ruled out entirely.1

Work concerning this question is in progress.

SUMMARY

In the presence of adenosine triphosphate and 3-phosphoglycerate disappearance of hydrolyzable P is observed in rabbit hemolysates and interpreted as formation of 2,3-P-GA.

¹ On the other hand, a biomolecular reaction mechanism, involving 3-P-GA as a coenzyme analogous to that of the phosphoglyceric mutase reaction (14) (suggested by Professor C. F. Cori in a personal communication), would lead to irreversible 2,3-P-GA formation.

A two-step reaction is assumed with formation of 1,3-P-GA, catalyzed by the phosphokinase of Bücher, preceding its transformation to 2,3-P-GA. The enzyme responsible for the second reaction, "diphosphoglycerate mutase," is absent from beef erythrocytes and muscle and is thought to be restricted in occurrence to red cells containing 2,3-P-GA. A partial purification of the enzyme has been achieved.

BIBLIOGRAPHY

- Greenwald, I., J. Biol. Chem., 63, 339 (1925). Rapoport, S., and Guest, G. M., J. Biol. Chem., 138, 269 (1941).
- 2. Negelein, E., and Broemel, H., Biochem. Z., 301, 135 (1939).
- Hevesy, G., and Aten, A. H. W., Kgl. Danske Vidensk. Selsk. Biol. Medd., 14, No. 5 (1939). Hevesy, G., and Hahn, L., Kgl. Danske Vidensk. Selsk. Biol. Medd., 15, No. 7 (1940).
- 4. Rapoport, S., and Guest, G. M., J. Biol. Chem., 129, 781 (1939).
- Braunstein, A. E., Biochem. Z., 272, 21 (1934). Schuchardt, W., and Vercellone, A., Biochem. Z., 272, 437 (1934).
- 6. Lennerstrand, R., Enzymologia, 9, 248 (1941).
- 7. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., 66, 375 (1925).
- 8. Rapoport, S., Biochem. Z., 289, 406 (1937).
- 9. Meyerhof, O., and Schultz, W., Biochem. Z., 297, 60 (1938).
- 10. Bücher, T., Biochim. et biophys. acta, 1, 292 (1947).
- 11. Rapoport, S., and Guest, G. M., J. Biol. Chem., 143, 671 (1942).
- 12. Meyerhof, O., and Oesper, P., J. Biol. Chem., 179, 1371 (1949).
- 13. Lipmann, F., Advances in Enzymol., 1, 105 (1941).
- 14. Sutherland, W., Posternak, T., and Cori, C., J. Biol. Chem., 181, 153 (1949).

CONVERSION OF LACTATE TO LIVER GLYCOGEN IN THE INTACT RAT, STUDIED WITH ISOTOPIC LACTATE*

By VICTOR LORBER, NATHAN LIFSON, HARLAND G. WOOD, WARWICK SAKAMI, AND WALTON W. SHREEVE†

WITH THE TECHNICAL ASSISTANCE OF JULIEN BORDEAUX AND MARGARET COOK

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, and the Department of Physiology, University of Minnesota Medical School, Minneapolis)

(Received for publication October 15, 1949)

The distribution of isotope in liver glycogen deposited following the feeding of a labeled compound may be used as an indicator in vivo of intermediary reactions linking the fed compound and glycogen. When acetate or butyrate labeled with isotopic carbon is administered together with non-isotopic glucose to a fasted rat, the resulting liver glycogen contains isotope (3, 4). If the distribution of the isotope in the glycogen is determined by hydrolysis and degradation of the resulting glucose, a number of characteristic patterns are observed (4). These distribution patterns are consistent with the reactions of the tricarboxylic acid cycle and of glycolysis as the main paths for the transfer of carbon from these non-carbohydrate sources to glycogen.

In the present experiments the metabolism of lactic acid has been studied by this technique. Three types of labeled lactate, ${}^{1}CH_{3} \cdot C^{18}HOH \cdot COONa$, $C^{18}H_{3} \cdot C^{18}HOH \cdot COONa$, and $C^{14}H_{3} \cdot C^{18}HOH \cdot COONa$ have been fed to fasted rats, and the resulting liver glycogen hydrolyzed to glucose and degraded. The distribution of isotope has been found to be in harmony with predictions based on the schemes of glycolysis and the tricarboxylic acid cycle. The results, when interpreted in terms of these schemes, indicate that the major fraction of the administered lactate car-

- * This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and by a grant to the Department of Biochemistry from the Prentiss Foundation. Preliminary accounts of some parts of this study have appeared (1, 2). The radiocarbon used in this work was obtained on allocation from the United States Atomic Energy Commission.
 - † Fellow of the American Cancer Society.
- ¹ Racemic mixtures of the D and L forms were fed in each case. Calculations involving the amount of isotope administered have been based on the isotope content of the total lactate fed, although it might have been more appropriate to consider only the isotope contained in the natural form. The possible metabolic rôle of the unnatural isomer in determining the results of the present experiments has otherwise been left out of consideration.

bon which was converted to glycogen traversed reactions of the tricarboxylic acid cycle prior to this conversion.

Methods

The lactate labeled with C^{13} in the α and β positions was synthesized according to the method of Cramer and Kistiakowsky (5) with some modifications. The C^{13} - α -labeled lactate was prepared from $CH_3 \cdot C^{13}OOH$ by esterification with butyl alcohol, and hydrogenation of the ester to the alcohols with copper-barium-chromium oxide catalyst, yielding $CH_3 \cdot C^{13}$ - H_2OH and $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$. The mixture of alcohols was converted to the corresponding iodides and fractionated. The ethyl iodide was converted by the Grignard reaction to propionic acid, $CH_3 \cdot C^{13}H_2 \cdot COOH$, which was isolated as the sodium salt. The final steps may be represented as follows:

 $CH_3 \cdot C^{13}H_2 \cdot COON \textbf{a} \ \underline{P \ + \ Br} \ CH_3 \cdot C^{13}HBr \cdot COOH \ \underline{Na_2CO_3} \ CH_3 \cdot C^{13}HOH \cdot COON \textbf{a}$

The C¹⁴H₃·C¹³HOH·COONa was prepared in a generally similar manner. C¹⁴H₃C¹³OOH was made from C¹⁴H₃I and KC¹³N. Conversion of the ethyl iodide to propionate was accomplished by nitrile formation and hydrolysis. The propionate was boiled with benzoyl chloride and the resulting propionyl chloride was removed by distillation. Bromination and hydrolysis yielded the isotopic lactate. Purity of the different acetic and propionic acids was checked by partition coefficient (6), which agreed with theory within experimental error. In all cases, the lactic acid was removed from the hydrolysis mixture by continuous ether extraction. Total acid, by titration, agreed with lactate determined colorimetrically (7).

The general plan of the experiments and the methods employed were similar to those described previously (4, 8). Male white rats, after a 24 hour fast, were given the isotopic lactate as the sodium salt by stomach tube, together with non-isotopic glucose to enhance glycogen deposition.² In several experiments, the respiratory CO₂ was collected. 3 hours after giving the lactate, the livers were removed under amytal anesthesia, and the glycogen isolated and hydrolyzed to glucose. When necessary, the glucose from more than one liver was pooled.

The glucose was converted to lactate by fermentation with Lactobacillus

² In some experiments preliminary to the present work, C^{13} - α,β -labeled lactate was administered in larger doses and without glucose. Not only were the glycogen yields much smaller, but the level of isotope in the glycogen was not much greater than that found in the present experiments. Comparable results were obtained by Vennesland *et al.* (9) who also administered α,β -labeled lactate without glucose. If its use is not contraindicated by other considerations, the administration of glucose thus permits economy of isotopic compound and makes for conveniently large glycogen yields with little decrease in isotope concentration in the glycogen.

casei, and the lactic acid was carried through a stepwise degradation to yield the carboxyl and α - and β -carbons of the lactate as separate fractions (4, 8). These fractions correspond to positions 3 and 4, 2 and 5, and 1 and 6 of the original glucose, respectively. In the experiments with $C^{14}H_3 \cdot C^{13}$ -HOH·COONa (Experiments 6 to 9) the fraction consisting of carbons 1 and 6, obtained as iodoform, was purified by sublimation before conversion to CO_2 in an effort to minimize dilution with adventitious, non-isotopic carbon, known to occur at this step (4). In addition, in Experiments 6 to 9 part of the glucose was converted to the methyl glucoside and degraded by an alternative procedure (4, 6). This degradation yields three separate fractions containing carbon 3, carbons 1, 2, 4, and 5, and carbon 6 of the glucose, respectively. The carbon 3 fraction is contaminated with small amounts of carbon from other positions. The various fractions

Table I
Degradation of Synthetic Isotopic Lactate*

. Type of lactate	Type of	Isotope concentration† in degradation fractions			
Type of factories	isotope	Carboxyl carbon	α-Carbon	β-Carbon	
CH ₃ ·C ¹³ HOH·COONa	C12 ('	0.00 0.01 0.00 0.00	0.58 1.14 1.34 25	0.00 1.16 0.00 2390	

^{*} The isotopic compounds were diluted with non-isotopic lactate before degrada-

from both types of degradation, after conversion to CO_2 , were analyzed for their C^{13} content in the mass spectrometer, as was the respiratory CO_2 . In Experiments 6 to 9 duplicate aliquots of all samples were precipitated as $BaCO_3$, and radioactivity determined with an end window Geiger-Müller tube. The method of sample preparation was very similar to that of Henriques *et al.* (10). The radioactivity measurement on the duplicate samples of the glycogen fractions agreed within ± 2 per cent. Duplicates on the respiratory CO_2 samples, which contained much less material, agreed within ± 5 per cent.

As a check on the synthesis and degradation procedures, the three types of synthetic lactate, after appropriate dilution with non-isotopic lactic acid, were degraded in the same manner as the biological material. The results, presented in Table I, show that the methods of synthesis and

[†] In this and subsequent tables, the C^{13} values are given in atom per cent excess, and the C^{14} values in counts per minute per mg. of carbon.

degradation are mutually confirmatory. A trace of C^{14} appears in the α -carbon fraction from the $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$.

RESULTS AND DISCUSSION

The general experimental data are summarized in Table II.

In Table III are presented the results of the glycogen degradations. It will be noted that for all types of lactate the isotope occurs predominantly in positions 1, 2, 5, and 6 of the glucose, and to a much lesser extent in carbons 3 and 4. In previous experiments on CO_2 fixation with labeled bicarbonate, positions 3 and 4 contained only about one-sixth the concentration of isotope found in the respiratory CO_2 (8), whereas in all the present studies for which data on respiratory CO_2 are available (Table IV) the

Table II
General Experimental Data on Rats

Experiment No.	Body weight after fast	Type of lactate administered	Amount of lactate adminis- tered per 100 gm. body weight	Amount of glucose adminis- tered per 100 gm. body weight	Liver weight	Liver glycogen (as glucose)
	gm.		m u	ты	gm.	ты
1	185	CH ₃ ·C ¹⁸ HOH·COONa	0.85	3.00	6.73	1.18
2	190		1.57	1.47	5.49	1 15
3	210	"	1.42	1.33	6.53	1.15
4	200	C18H2 · C18HOH · COONa	1.60	1.40	6.65	0.96
5	180	"	1.78	1.56	5.73	0.83
6	225	C14H2 · C18HOH · COONa	1.96	2.17	7.30	1.33
7	190	"	1.89	2.10	5.00	0.80
8	205	"	1.85	2.06	5.50	0.07
9	205	"	1.81	2.01	6.00	0.87

relative concentration runs appreciably higher, being about equal to the respiratory CO₂ in Experiment 5. Although the two types of experiment are not strictly comparable, since the labeled CO₂ is being produced metabolically in the present studies, it seems very unlikely that the level of isotope appearing in carbons 3 and 4 in the lactate experiments can be accounted for by CO₂ fixation alone.

In the experiments with lactate containing C^{18} only in the α position (Experiments 1 to 3 and 6 to 9), carbons 2,5 of the glucose are seen to contain about one-third more C^{18} than carbons 1,6. When the β -carbon is labeled with C^{14} (Experiments 6 to 9), the relative distribution of isotope between these positions is reversed, although the disparity between carbons 1,6 and 2,5 is not as great as that noted for the C^{18} of the α -carbon.

Table III

Distribution of Isotope* in Liver Glycogen after Administration of Labeled Lactate to
Rats

Experiment No.	Type of lactate administered		Isotope concentration in labeled carbon of administered lactate	Isotope concentration in degradation fractions of glucose Carbon atoms of glucose		Isotope concentration in degradation fractions of methyl glucoside Carbon atoms of glucose			er cent of adminis- tered isotope recov- ered in liver glycogen	
Expe	•	Type	Isote in adu	3,4	2,5	1,6	3	1,2, 4,5	6	Per ter ere
1	CH ₃ ·C ¹³ HOH·COONa	C18	4.64	0.01	0.10	0.07				5.91
2-3	"	"	4.64	0.05	0.27	0.18				4.23
4	C13H3 · C13HOH · COONa	"	5.06	0.15	0.60	0.59				8.1
5	"	"	5.06	0.18	0.94	0.89				10.8
6†	C14H3 · C13HOH · COONa	"	6.50	0.07	0.42	0.30				7.36
		C14	11,600	204	679	826				8.93
6-9	· ·	C13	6.50	0.10	0.43	0.30	0.09	0.30	0.29	4.86‡
		C14	11,600	201	681	742	227	577	756	5.54‡

^{*} Sec foot-note to Table I.

Table IV

Data on Respiratory CO₂ of Rats

Ex- peri- ment No.	Type of lactate administered	CO2 output in mm			Type of	Isotope in re	Per cent of admin- istered isotope		
		1st hr.	2nd hr.	3rd hr.	tope	1st hr.	2nd hr.	3rd hr.	in res- piratory CO ₂
2	CH2·C13HOH·COONa	7.82	5.83	6.44	C^{18}	0.10	0.15	0.08	15.7
5	C18H2 · C18HOH · COONa	7.19	6.64	6.82	"	0.13	0.17	0.21	10.8
7	C14H2 · C18HOH · COONa	6.99	7.23	6.76	"	0.08	0.24	0.28	17.9
					C14	143	351	476	16.2
8	"	6.69	5.82	6.46	C_{13}	0.05	0.13	0.20	9.64
					C14	92	243	318	9.29

^{*} See foot-note to Table I.

The results obtained by the two degradation procedures in Experiments 6 to 9 are seen to be in good agreement. The values for carbon 6 from the degradation of the methyl glucoside agree, within experimental error, with the values for carbons 1,6 from the glucose degradation. The expected

^{† 1} mm of glucose was degraded. The remaining 0.33 mm was combined with that from Experiments 7 to 9, and the pooled sample divided equally and degraded by both methods.

[‡] These calucations are based on the total isotope administered and the total recovered in liver glycogen in Experiments 6 to 9.

concentrations for the fraction containing positions 1, 2, 4, and 5, calculated from the results for the three fractions of the glucose degradation, are 0.31 atom per cent excess and 576 c.p.m. per mg. of carbon for C¹³ and C¹⁴ respectively, in agreement with experimental values of 0.30 and 577. Agreement between carbons 3,4 and 3 is satisfactory, in view of the low C¹³ values and the fact that the carbon 3 fraction contains a small amount of material from other positions.

With the C^{13} - α , β -labeled lactate, the isotope values for carbons 2,5 and 1,6 in Experiment 4 are equal, within the error of the measurement (± 0.01 atom per cent). In Experiment 5, positions 2,5 appear to contain about 5 per cent more C^{13} than positions 1,6. This is due to a procedural artifact. As pointed out previously (4) the degradation procedure as usually employed causes a certain amount of dilution of fraction 1,6 with non-isotopic CO_2 . This dilution produces a depression of the isotope values for this fraction, which is most marked when the samples are small and when the isotope values are high. In Experiments 2 to 3, at the relatively low C^{13} value of 0.18 atom per cent excess, and large sample size of 0.84 mm, the dilution effect is masked. In Experiment 5, at a higher C^{13} value and smaller sample size (0.89 atom per cent excess and 0.45 mm, respectively), the dilution effect becomes apparent. These dilution effects have been observed by degradation of synthetic lactate, equally labeled with C^{13} in the α and β positions (4).

The data obtained by the simultaneous use of C13 and C14 in Experiments 6 to 9 permit fairly accurate comparison of the relative contribution of the α - and β -carbons of the lactate to liver glycogen and respiratory CO₂. Since the C13 and C14 are administered together in the same compound, and determined in aliquots of the same degradation fractions, the significance of any differences noted will not be influenced by variations between animals or chemical procedures, but will depend solely on the errors involved in the isotopic measurements. The values for the per cent of administered isotope recovered in liver glycogen in these experiments, presented in the last column of Table III, indicate that, although the transformation of α - and β -carbons to glycogen is of the same order of magnitude, a larger contribution has been made by the β -carbon. The observed differences lie beyond the errors of the measurements. (In similar experiments with C14H3. C18H2. COONa in which, unlike the present experiments, C18 and C¹⁴ were found to be equally distributed between carbons 2,5 and 1,6 of the glucose, the per cent recovery in the glycogen was identical for both isotopes (11).) In the last column of Table IV (Experiments 7 and 8) it will be noted that the per cent of administered isotope recovered in the respiratory CO2 is approximately the same for both isotopes, indicating that the α - and β -carbons of lactate have contributed about equally to the respiratory CO₂. It might be expected that the introduction of a relatively larger amount of one isotope in the glycogen would result in the appearance of a relatively smaller amount of this isotope in the respiratory CO₂. The differences between the per cent of C¹⁸ and C¹⁴ recovered in the respiratory CO₂, while compatible with this expectation both in direction and magnitude, lie within the error of the isotopic measurements on the respiratory CO₂ samples and cannot be considered as significant.

On the assumption that glycogen is formed by reversal of the glycolytic reactions as indicated in Diagram 1 (Reactions 2 and 1), the main results of the present study indicate that isotope administered in either the α - or β -carbon of lactate is largely, though not completely, randomized between these positions in pyruvate, and that, when isotope is introduced in equal concentration in both the α - and β -carbons of lactate simultaneously, the concentration remains equal in the resulting pyruvate.

The reactions of glycolysis and the tricarboxylic acid cycle, presented in abridged fo m in Diagram 1,3 readily account for the main results. On the assumption that lactate enters into metabolism via pyruvate, reversal of the glycolytic reactions will lead to the following types of isotopic glucose,

C—C—C—C—C or C—C—C—C—C—C, depending on whether the isotope is introduced in the α - or β -carbon of the lactate (the asterisk indicates the location of the isotope). CO₂ fixation and the reversible reductive reactions of the tricarboxylic acid cycle (Reactions 3, 10, 9, and 8) followed by Reaction 2, will produce a glucose with isotope equally

distributed in carbons 1, 2, 5, and 6 (C—C—C—C—C—C), regardless of whether the isotope is introduced in the α or β position of the lactate.

The contribution to the isotopic "pyruvate pool" by decarboxylation of oxalacetate formed via the oxidative reactions of the cycle must also be considered. The oxidative reactions of the tricarboxylic acid cycle (Reactions 5 to 10) will involve initially an isotopic oxalacetate (Reaction 3) as well as an isotopic 2-carbon compound (Reaction 4). In the case of C¹⁴H₃·C¹³HOH·COONa, COOH·C¹⁴H₂·C¹³O·COOH and C¹⁴H₃·C¹³OX will be the initial reactants. Assuming that the initial condensation reaction occurs on the carbonyl group of oxalacetate, as usually depicted, the newly

*Stern and Ochoa (12) have recently demonstrated citrate accumulation in an aged pigeon liver preparation, using acetate and oxalacetate together in substrate amounts. The preparation was very low in aconitase activity, pointing to citrate as the initial condensation product in the enzyme system involved. Because of this and other recent developments (13, 14), which emphasize the tentative nature of current schemes of the tricarboxylic acid cycle, the individual tricarboxylic acids have been omitted from Diagram 1.

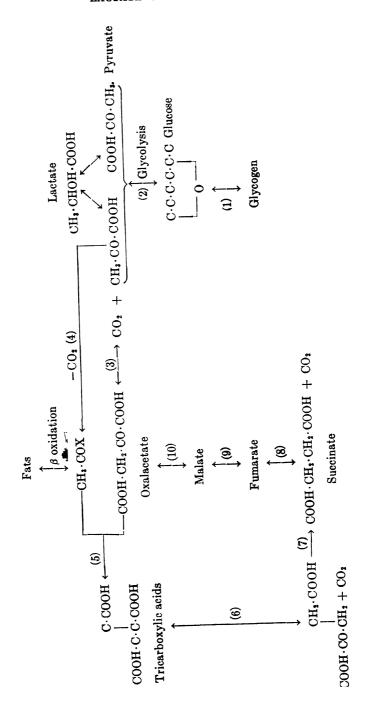
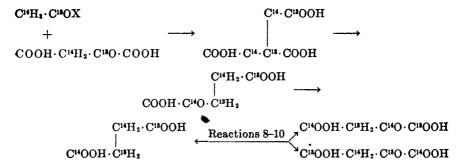


DIAGRAM 1. Abridged tricarboxylic acid cycle and its relationship to glycogen synthesis α -Ketoglutarate

formed oxalacetate generated via the cycle will contain both isotopes in all positions, since, according to the scheme of Diagram 1,



Any of this oxalacetate going to glycogen via Reactions 3 to 1 will introduce both isotopes into all positions of the hexose chain. C¹² will be equally distributed among carbons 1, 2, 5, and 6, as will C¹⁴. However, the relative abundance of the two isotopes in these positions will depend upon the isotope dilution occurring in the "acetate" and "oxalacetate pools," respectively. For example, heavier labeling in the "acetate pool" should yield a hexose relatively richer in C¹⁴ than in C¹³. In addition, under this latter condition, the disparity in isotope concentration between positions 1,6 and 2,5 of the final glycogen, produced by direct conversion of C¹⁴H₃·C¹³HOH·COONa via glycolysis, should be less pronounced in the case of C¹⁴, as was actually found.

It will be noted that the oxidative reactions of the tricarboxylic acid cycle provide a path (in addition to CO_2 fixation) for the transfer of isotope from the α - and β -carbons of pyruvate to positions 3,4 of glucose. It may be wondered why the relative concentration of isotope in carbons 3,4 of the glucose is not higher than that actually found in the present study. It should be pointed out that in the presence of a respiratory CO_2 low in isotope, as in the present experiments, the CO_2 fixation reaction may operate to dilute the isotope in carbons 3,4 of the glucose. The contrary would be expected when the respiratory CO_2 is relatively high in isotope, and actually it has been found in similar experiments with C^{13} - $H_3 \cdot C^{13}OONa$ (4), in which the isotope level in the respiratory CO_2 was several times that obtaining in the present study, that the C^{13} concentration in carbons 3,4 exceeded that in the other positions of the glucose.

Study of the schemes presented in Diagram 1 will also reveal that there is no mechanism for altering complete randomness of distribution of isotope between the α - and β -carbons of pyruvate, once it has been established. As noted (Table III) in the experiments with C¹²H₃·C¹³HOH·COONa, the resulting liver glycogen contains C¹³ equally distributed

between the 1,6 and 2,5 fractions, in accordance with expectations. The reactions of Diagram 1 require, furthermore, that isotope administered in the carboxyl position of lactate should occur exclusively in positions 3 and 4 of the resulting liver glycogen. In experiments with CH₃·CHOH·C¹³OONa, undertaken in another connection, it has been noted that isotope was present in detectable amounts only in carbons 3 and 4, as expected.

Some idea of the relative amount of isotope that traverses the reactions of the tricarboxylic acid cycle prior to conversion to glycogen via Reactions 2 and 1 may be gained from a consideration of the distribution of isotope in the glycogen in experiments in which α -labeled lactate has been fed. (Similar considerations would apply to the experiments in which β -labeled lactate was administered.) Glucose formed via Reaction 2, before randomization of the isotope has occurred, will exhibit the following

isotope distribution pattern, C—C—C—C—C—C, in the case of α -labeled lactate. As pointed out earlier, CO₂ fixation and the reductive reactions of the tricarboxylic acid cycle (Reactions 3, 10, 9, and 8), followed by Reaction 2, will distribute isotope equally between carbons 1,6 and 2,5 of

the resulting glucose (C—C—C—C—C). The CO₂ fixation reaction and the oxidative reactions of the cycle will introduce isotope into carbons 3,4 of the glucose, but will not disturb the equality of distribution between fractions 1,6 and 2,5. The final glycogen in experiments with α -labeled lactate may therefore be considered as having been constituted from es-

sentially two types of isotopic glucose, C—C—C—C—C, and C—C—C—C—C—C. All of the isotope in the second type of glucose, according to the proposed schemes, must have been involved in one way or another in Reactions 3 to 10.

Let us take as an example the distribution of isotope in the liver glycogen in Experiment 6 (Table III). The hexose chain may be pictured 0.30 0.42 0.07 0.07 0.42 0.30

thus, C—C—C—C—C—C, in which notation the superscripts are to be taken as representing absolute amounts of C¹⁸ in each position in the molecule, rather than isotope concentrations. The two types of isotopic glucose from which the final product was constituted may be 0.12 0.30 0.30 0.07 0.07 0.30 0.30

represented as C—C—C—C—C—C—C—C—C—C—C—C—C. Thus

⁴ The symbols indicate the relative isotope concentration, $* > \circ$.

⁵ For purposes of comparison, the absolute amounts of isotope in the different positions of the glucose may be represented by the values for concentration, since atom per cent excess $C^{13} \times (\text{mm of glucose})/100 = \text{mm excess } C^{13}$ for any given position in the glucose, and the factor (mm glucose)/100 is common to the calculation for all positions.

it appears that the amount of C¹⁸ that has been involved in the reactions of the tricarboxylic acid cycle prior to deposition in glycogen is about 5 times as great (1.34 to 0.24) as that which has gone directly to glycogen via glycolysis. If the C¹⁴ data from the same experiment are handled similarly, a ratio of the order of 10:1 is obtained. It should be borne in mind that the differences in isotope content between carbons 1,6 and 2,5, on which these ratios are based, are not very large compared to the errors involved in estimating the isotope concentrations.

While the above figures may give an approximate indication of the relative amount of isotope that entered glycogen by different routes in the present studies, they need not indicate the relative amount of glycogen constituted from precursors coming over the different paths, since the degree of isotope dilution may not be the same for the different paths. Indeed, it seems quite reasonable to assume a lesser degree of dilution for isotope traversing the direct pathway of Reactions 2 and 1, from which it follows that the calculated ratios give a minimum value for the relative amount of glycogen formed from precursors that have been involved in the tricarboxylic acid cycle compared with those that have taken the direct path. In other words, a molecule of pyruvate introduced into the liver "pyruvate pool" should have, on the average, at least five chances to enter the reactions of the tricarboxylic acid cycle for every chance for entry into the glycolytic reactions (assuming that the reactions of glycolysis are essentially one way in the direction of glycogen deposition, under the circumstances obtaining). The recent paper of Topper and Hastings (15), in which the distribution of isotope in glycogen deposited in liver slices, with α -labeled pyruvate as substrate, is reported, is confirmatory, in general, of the corresponding experiments performed in the intact animal, as described in the present study and in a preliminary report (1). Topper and Hastings have calculated that involvement of pyruvate in CO₂ fixation and subsequent reactions occurs 4 times as rapidly as does entry into the reactions leading from pyruvate to glycogen. The presentation of the rôle of the tricarboxylic acid cycle in the distribution of isotope in glycogen is somewhat incomplete, since there is no discussion of the possibility that the cycle may act as a pathway, in addition to CO₂ fixation, for introducing isotope into positions 3 and 4 of the glucose. Also the possibility that there may be differences in the degree of isotope dilution due to side reactions occurring on the different metabolic pathways is not considered. It may be, of course, that this latter factor is of little importance in determining the results in experiments of this type. Its rôle, however, remains to be evaluated.

It should be pointed out that the foregoing discussion has assumed, implicitly, a homogeneous metabolic system in which mutual interaction

between the different metabolic pathways could occur freely. This is obviously not the case in the intact rat. Nevertheless, it seems likely that, in the present experiments, the isotopic composition of liver glycogen reflects, in the main, metabolic events within the liver cell, where the assumption of homogeneity may more nearly apply. The isotopic composition of the respiratory CO₂, on the other hand, must result from the summed activity of all the tissues of the animal's body.

Finally, it should be pointed out that the general argument, presented in terms of current metabolic schemes, has been conducted in a rather dogmatic fashion in order to limit the discussion to a reasonable length. The general agreement between the results presented and these schemes enhances the likelihood of their reality in the intact animal, but cannot be considered as establishing them as the main or sole metabolic pathway involved in the present studies, as might be inferred if some sections of the discussion are construed too literally.

SUMMARY

CH₃·C¹⁸HOH·COONa, C¹⁸H₃·C¹⁸HOH·COONa, and C¹⁴H₃·C¹³HOH·COONa have been fed to rats and the resulting liver glycogen isolated and degraded, and the distribution of isotope in the glucose chain determined. The results have been interpreted in terms of the reactions of glycolysis and the tricarboxylic acid cycle, with which schemes they appear to be in general agreement. The results with lactate labeled in the α - or β -carbon indicate that of the administered lactate carbon that was deposited as liver glycogen probably less than one-sixth entered the glycogen directly via the reactions of glycolysis, without prior passage through reactions involving randomization of isotope between the α - and β -carbons.

BIBLIOGRAPHY

- Lorber, V., Lifson, N., Wood, H. G., and Sakami, W., Am. J. Physiol., 155, 452P (1948).
- 2. Wood, H. G., Cold Spring Harbor Symposia Quant. Biol., 13, 201 (1948).
- Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., J. Biol. Chem., 150, 413-(1943).
- Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., J. Biol. Chem., 176, 1263-(1948).
- 5. Cramer, R. D., and Kistiakowsky, G. B., J. Biol. Chem., 137, 549 (1941).
- Osburn, O. L., Wood, H. G., and Werkman, C. H., Ind. and Eng. Chem., Anal. Ed., 8, 270 (1936).
- 7. Barker, S. B., and Summerson, W. H., J. Biol. Chem., 138, 535 (1941).
- 8. Wood, H. G., Lifson, N., and Lorber, V., J. Biol. Chem., 159, 475 (1945).
- Vennesland, B., Solomon, A. K., Buchanan, J. M., Cramer, R. D., and Hastings, A. B., J. Biol. Chem., 142, 371 (1942).

- Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., Ind. and Eng. Chem., Anal. Ed., 18, 349 (1946).
- Lorber, V., Lifson, N., Sakami, W., and Wood, H. G., J. Biol. Chem., 183, 531 (1950).
- 12. Stern, J. R., and Ochoa, S., J. Biol. Chem., 179, 491 (1949).
- 13. Ogston, A. G., Nature, 162, 963 (1948).
- 14. Potter, V. R., and Heidelberger, C., Nature, 164, 180 (1949).
- 15. Topper, Y. J., and Hastings, A. B., J. Biol. Chem., 179, 1255 (1949).

CONVERSION OF PROPIONATE TO LIVER GLYCOGEN IN THE INTACT RAT, STUDIED WITH ISOTOPIC PROPIONATE*

By VICTOR LORBER, NATHAN LIFSON, WARWICK SAKAMI, AND HARLAND G. WOOD

WITH THE TECHNICAL ASSISTANCE OF MARGARET COOK AND JULIEN BORDEAUX

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, and the Department of Physiology, University of Minnesota Medical School, Minneapolis)

(Received for publication, October 15, 1949)

It has been found that when various fatty acids containing an odd number of carbon atoms in the chain (C₃, C₅, C₇, C₉ acids) are fed to fasted rats deposition of liver glycogen occurs, which is of approximately the same magnitude for each acid (4). The feeding of valeric, heptylic, pelargonic, and undecyclic acids to well fed rabbits leads to an increase in blood acetone bodies, while the administration of propionic acid does not (5). Similarly, incubation of the C₅, C₇, and C₉ acids with liver slices has been found to result in acetoacetate formation, which did not occur when propionate was used as the substrate (6). On the basis of these findings the odd carbon fatty acids have been considered as undergoing successive β oxidation, with the formation of propionate from the terminal 3-carbon residue. Recently Atchley (7) has demonstrated the formation of propionate in a kidney enzyme preparation, using valerate as the substrate. The fate of propionate itself in the animal body, however, is far from established. Propionate given to phlorhizinized dogs (8) and fasted rats (4) leads to the excretion of extra glucose and the deposition of liver glycogen, respectively. While these findings may be accepted as evidence for the conversion of propionate to carbohydrate, the chemical pathway for this transformation remains to be defined.

^{*} This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and by a grant to the Department of Biochemistry from the Prentiss Foundation. Preliminary reports of some parts of this work have appeared (1-3). The radiocarbon used in this work was obtained on allocation from the United States Atomic Energy Commission.

Hahn and Haarmann (9) have reported the accumulation of pyruvate in a muscle mince incubated with propionate and have suggested the accompanying series of reactions to account for their finding.

Bloch and Rittenberg (10), using deuterium as a labeling agent, have examined the ability of a variety of compounds to acetylate fed foreign amines. Propionate and alanine were studied, the latter being considered equivalent to pyruvate metabolically. On the basis of the deuterium content of the excreted acetyl groups, alanine was judged to be an efficient acetylating agent, while propionate was not. This was considered as evidence against the conversion of priopionate to pyruvate.

The present study, with use of carbon-labeled propionate, was undertaken in an effort to gain more information concerning the fate of propionate in the animal body. Variously labeled isotopic propionates, plus glucose, were fed to fasted rats, and, after a suitable period of time, the livers were removed, the glycogen isolated and hydrolyzed to glucose, and the distribution of isotope in the glucose chain determined. Although the observed isotope distribution patterns have been found to bear a basic resemblance to those obtained with similarly labeled lactate (11), a finding pointing to pyruvate as an intermediate in propionate metabolism, there are certain differences between the two sets of patterns which indicate that a direct conversion to pyruvate or lactate may not occur.

Methods

The following compounds were studied: $CH_3 \cdot CH_2 \cdot C^{13}OONa$, $C^{13}H_3 \cdot CH_2 \cdot COONa$, and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$. The carboxyl-labeled compound was synthesized by the Grignard reaction. The β -labeled propionate was prepared from $C^{13}H_3 \cdot COOH$ (3), and the C^{14},C^{13} -labeled compound from $C^{14}H_3 \cdot C^{13}OOH$ (11). The labeled acetates were reduced to ethanol by catalytic hydrogenation, then converted to ethyl iodide, and the corresponding propionate prepared via nitrile formation and hydrolysis (11). The partition coefficient (12) was determined for each of the three isotopic propionates and agreed with established values within experimental error. Conversion of the C^{14}, C^{13} -propionate to lactate, and stepwise degradation of the lactate by well established procedures, showed the isotope to be distributed as anticipated from the synthetic methods employed (11).

The general plan of the experiments and the methods employed have been fully described in prior communications (13, 14, 11). In the experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, the glucose from the liver glycogen was degraded by the two types of procedure previously described (13).

RESULTS AND DISCUSSION

The general experimental data are summarized in Table I. In Table II are presented the results of the glycogen degradations. The data from

the experiments with $C^{18}H_2 \cdot CH_2 \cdot COONa$ and $C^{14}H_3 \cdot C^{18}H_2 \cdot COONa$, when consideration is given to the known errors in the degradation methods (14), show that isotope originating in the α - and β -carbon of propionate is completely randomized between the 1,6 and 2,5 positions of glucose. The errors in the present degradation methods, as pointed out previously (14), are that fraction 1,6 is usually subject to contamination with a small amount of non-isotopic carbon, and that the fraction for carbon 3 is not entirely free of carbon from other positions. Carbon 6, from the methyl glucoside degradation, is obtained by a series of fairly specific reactions (13), and appears to be free from such contamination. On the assump-

Table I
General Experimental Data from Rats

Experiment No.	Body weight after fast	Sex	Type of propionate administered	Amount of pro- pionate adminis- tered per 100 gm body weight	Amount of glucose adminis- tered per 100 gm. body weight	Liver weight	Liver glycogen (as glucose)
	gm.			m M	ты	gm.	mM
1	240	ď	CH2·CH2·C18OONa	2.60	2.32	7.2	0.19
2	210	₫	"	3.00	2.65	6.3	0.27
3	196	Q	C18H3 · CH2 · COONa	2.12	3.53	4.6	0.56
4	182	Q	"	2.10	3.50	5.7	0.50
5	188	Ş	"	2.10	3.50	6.0	0.52
6	197	Ç	"	2.11	3.51	6.7	0.23
7	210	∂"	C14H3. C18H2. COONa	2.51	3.32	6.6	1)
8	230	♂	"	2.54	3.37	7.0	5.19
9	210	♂	"	2.54	3.37	7.3	5.19
10	240	∂ਾ	"	2.66	3.52	8.4])
11	190	ਰਾ	"	2.70	3.54	6.6	1)
12	220	ď	46	2.66	3.52	7.0	3.98
13	230	♂	"	1.92	2.55	7.2	J

tion that the hexose chain is formed from 2 triose molecules of similar isotopic composition, carbon 6 may be taken as a measure of the isotope content of fraction 1,6. It will be noted that, in Experiments 7 to 10 and 11 to 13, both the C¹³ and C¹⁴ values for carbon 6 are in excellent agreement with the values obtained for carbons 2,5. Likewise there is good agreement between the experimentally determined isotope concentrations in the carbon 1, 2, 4, and 5 fraction of the methyl glucoside degradation and the values calculated for this fraction (cf. Table II). The over-all results are therefore taken to indicate that the isotope concentrations in the 1, 2, 5, and 6 positions are equal.

Calculations of the per cent of administered isotope recovered in the

1, 2, 5, and 6 carbons of the glucose, presented in parentheses in the last column of Table II, yield values for C^{13} and C^{14} which are identical within experimental error, as might be expected for a mechanism of conversion of propionate to glucose which involved complete randomization of the α - and β -carbons of the propionate between the 1,6 and 2,5 carbons of the glucose.

Table II

Distribution of Isotope* in Liver Glycogen after Administration of Labeled Propionate
to Rats

Exper- iment No.	Type of propionate administered	Type of isotope	Isotope concentra- tion in labeled po- sition of adminis- tered propionate	tratio datio	ope con in con fracti gluco	legra- tions se toms	fract	cope conce in degrad tions of m glucoside rbon atom glucose	ation ethyl	adm isos cos	cent of inistered tope re- vered in liver ycogen
1 2 3- 6 7-10		C ₁₃	$\frac{4.62}{2.28}$		0 0 17	01 .01 0.16 0.35		0 32	0.39	0.2 0.2 2.64 5.93	(5.31)‡
11-13	u	C18	11,600 6.50 11,600	0.08	0.41	0.37		548 (558)†	691 0.41	7.17	(5.35)‡ (6.54)‡ (6.68)‡

^{*} The C¹⁸ values are given in atom per cent excess, and the C¹⁴ values in counts per minute per mg. of carbon.

As previously reported (11), when $C^{14}H_3 \cdot C^{13}HOII \cdot COONa$ is fed to a fasted rat, the α - and β -carbons of the lactate are largely, though not completely, randomized between the 1,6 and 2,5 carbons of the glucose from the resulting glycogen. The distribution is, in general, the same as that noted in the present experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, except that in the case of $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ C^{18} is present in somewhat higher concentration in carbons 2,5 than in carbons 1,6, while C^{14} is more abundant in the latter fraction than in the former. The incomplete

[†] The figures in parentheses are calculated values, in which the isotope concentration for carbon 4 is taken as equal to fraction 3,4 and that in carbons 1, 2, and 5 is taken as equal to fraction 2,5.

[‡] The values in parentheses represent the per cent of administered isotope recovered in carbons 1, 2, 5, and 6, the isotope values for the 2,5 fraction being taken as representative of all 4 carbon atoms.

randomization, in the case of lactate, has been explained in terms of the reactions of glycolysis and the tricarboxylic acid cycle. The fact that complete equalization does not occur has been ascribed to conversion to pyruvate and direct incorporation of some of the pyruvate into glycogen, prior to randomization of the isotope via the tricarboxylic acid cycle (11).

If one assumes that pyruvate is an obligatory precursor of glucose and glycogen, the isotope distribution patterns observed in the glycogen in the present studies indicate that propionate labeled in the α - or β -carbon gives rise to pyruvate in which both α - and β -carbons contain equal labeling.

In view of the results with labeled lactate (11), this may be taken to mean that propionate cannot be converted more or less directly to pyruvate (as, for example, via acrylate and lactate (9)), but that a more complex mechanism involving complete randomization of the α - and β -carbons intervenes. What this mechanism might be is a matter of conjecture. One possibility may be indicated in simplified form. Carboxylation on the methyl group of propionate would lead to symmetrical succinate. From such succinate (via fumarate, malate, and oxalacetate), pyruvate would be formed containing isotope from the α or β position of propionate equally distributed between both the α - and β -carbons. However, the authors are aware of no evidence for such a reaction in mammalian tissues. The reverse reaction, the formation of CO₂ and propionate by the decarboxylation of succinate, has been described in certain microorganisms (15-17). It is not meant to suggest that the statement, succinate \rightarrow propionate + CO₂, describes anything but the net result of what may be a series of reactions. Similarly, it is intended only to point out that an over-all conversion of propionate to succinate by a C₁ + C₃ addition would account for the isotopic data (as well as for the glycogenic properties of propionate).

On the other hand, a more direct path from propionate to pyruvate may exist, but, because of differences in reaction rates, the major part of the propionate carbon traverses an indirect pathway to pyruvate, involving randomization of isotope. A hypothetical example is presented in the accompanying diagram.

Glycogen

$$\downarrow \uparrow$$
Pyruvate $\rightleftarrows X \xleftarrow{+CO_2} \xrightarrow{-CO_2}$ oxalacetate \rightleftarrows malate, fumarate, etc.

Propionate

Propionate is depicted as being converted without randomization to an intermediate, X, lying between pyruvate and the reactions of the tricarboxylic acid cycle. If the reaction rate from X toward the randomizing reactions of the tricarboxylic cycle is sufficiently greater than that from X toward pyruvate, isotope from the α - or β -carbons of propionate could be fairly completely randomized between the α and β positions of the pyruvate formed from X. The only available evidence bearing on this suggestion is the finding by Utter and Chenoweth (18) that pyruvate may not be the immediate reactant involved in the CO₂ fixation reaction by which oxalacetate is formed.

Per cent CO2 output Isotope concentrationt of adminiain respiratory CO2 tered iso-Type of Type of propionate Experitope recov-ered in ment No. administered isotope 2nd 3rd 1st 2nd3rd respiratory 1st per-iod perper-COı period period period iod ind CH2 · CH2 · C13OONa 8.7 8.5 7.9 C18 1 0.120.210.2316.67.2 6.2 6.3 " 0.250.2713.1 2 0.133-4 C18H3. CH2. COONa 12.4 | 12.2 | 14.80.080.080.1019.3 13.7 14.2 13.9 " 0.06 0.09 0.08 17.4 5-6 C14H2 · C18H2 · COONa 8.9 8.5 " 0.220.2615.2 0.12 C^{14} 232 473 518 17.3 C13 8 7.9 $7.5 \mid 8.5 \mid$ 0.130.170.078.6 C14 218 369 188 9.4

TABLE III

Data on Respiratory CO₂ of Rats

It is interesting to note that the degree of isotope dilution undergone by the $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$ in its conversion to liver glycogen is of the same order as that found for $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ (11). This together with the observed isotope distribution patterns indicates that propionate carbon gives rise to pyruvate (assuming pyruvate to be an obligatory intermediate in glucose and glycogen formation), and at about the same rate as lactate. That the findings of Bloch and Rittenberg using deuterium-labeled propionate in isotope dilution experiments (10), as pointed out earlier, are not in agreement with the foregoing conclusion may have been due to the loss of deuterium from the propionate in the same reactions which bring about the complete randomization of the α -and β -carbons of propionate in the 1, 2, 5, and 6 positions of the glucose in the present experiments.

^{*} The CO₂ collection periods were 50 minutes, except for Experiments 7 and 8, in which they were 1 hour.

[†] See foot-note to Table II.

Some of the other experimental results obtained in the present study are worthy of note. The data for respiratory CO2 in Experiments 7 and 8 (Table III) indicate that the α - and β -carbons of propionate are converted to CO₂ at approximately equal rates. Although the differences between the rate of recovery of C18 and C14 in the respiratory CO2 appear fairly large, they are not significant because of the relatively larger error of the isotopic measurements on the respiratory CO2 samples, owing particularly to the low C13 levels in the respiratory CO2. It may be noted, incidentally, that the glycogen degradation data in the experiments with CH₃·CH₂·C¹⁸-OONa and C18H₃·CH₂·COONa rule out β oxidation to malonate with the formation of acetate (or an equivalent 2-carbon compound) from the α and carboxyl carbons of malonate as an important pathway in propionate metabolism. Since both types of propionate would yield carboxyl-labeled malonate, and consequently a carboxyl-labeled 2-carbon compound, this mechanism should deposit isotope exclusively in positions 3 and 4 of the glucose from liver glycogen following feeding of both types of propionate (14). The finding of label predominantly in the 1,6 and 2,5 carbons in the experiments with the β -labeled propionate cannot be reconciled with this pathway.

It should be clear that the foregoing discussion cannot be generalized to extrahepatic tissues, in which reactions may occur that will not affect the isotope distribution in liver glycogen. In this connection it should be pointed out that in many instances in the present and in former experiments with other labeled compounds glycogen of cardiac and skeletal muscles has been isolated and degraded. Isotope concentrations have always been much lower than those found in liver glycogen, but the distribution patterns have always been the same. While this may indicate basically similar metabolic pathways in liver and muscle for the compounds concerned, it may also be nothing more than a reflection of hepatic metabolism via the blood sugar.

SUMMARY

Isotopic propionate (CH₃·CH₂·Cl³OONa, Cl³H₃·CH₂·COONa, and Cl⁴-H₃·Cl³H₂·COONa) has been fed to fasted rats and the resulting liver glycogen isolated, hydrolyzed to glucose, and degraded. Isotope from the carboxyl group of propionate has been found to give detectable labeling only in carbon atoms 3 and 4 of the glucose, while propionate containing isotope in the α or β position gives rise to glucose labeled predominantly and equally in carbons 1, 2, 5, and 6.

If it is assumed that pyruvate is an obligatory intermediate in glucose and glycogen formation, the results indicate that propionate gives rise to pyruvate, but that in the process the α - and β -carbons of the propionate

lose their orientation and become equally distributed between the corresponding groups of pyruvate. Some possible explanations for this finding are advanced.

It is a pleasure to acknowledge the help of Dr. Walton W. Shreeve in several of the experiments.

BIBLIOGRAPHY

- Lorber, V., Lifson, N., Sakami, W., and Wood, H. G., Federation Proc., 6, 272 (1947).
 Lorber, V., Cook, M., and Bordeaux, J., Federation Proc., 8, 99 (1949).
- Wood, H. G., in A symposium on the use of isotopes in biology and medicine, Madison, 209 (1948).
- 3. Wood, H. G., Cold Spring Harbor Symposia Quant. Biol., 13, 201 (1948).
- Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., and Cutler, C. H., J. Biol. Chem., 112, 15 (1935-36).
- 5. MacKay, E. M., Wick, A. N., and Barnum, C. P., J. Biol. Chem., 136, 503 (1940).
- 6. Jowett, M., and Quastel, J. H., Biochem. J., 29, 2159 (1935).
- 7. Atchley, W. A., J. Biol. Chem., 176, 123 (1948).
- 8. Ringer, A. I., J. Biol. Chem., 12, 511 (1912).
- 9. Hahn, A., and Haarmann, W., Z. Biol., 90, 231 (1930).
- 10. Bloch, K., and Rittenberg, D., J. Biol. Chem., 155, 243 (1944).
- Lorber, V., Lifson, N., Wood, H. G., Sakami, W., and Shreeve, W. W., J. Biol. Chem., 183, 517 (1950).
- Osburn, O. L., Wood, H. G., and Werkman, C. H., Ind. and Eng. Chem., Anal. Ed., 8, 270 (1936).
- 13. Wood, H. G., Lifson, N., and Lorber, V., J. Biol. Chem., 159, 475 (1945).
- Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., J. Biol. Chem., 176, 1263 (1948).
- 15. Werkman, C. H., and Wood, H. G., Advances in Enzymol., 2, 135 (1942).
- 16. Delwiche, E. A., J. Bact., 56, 811 (1948).
- 17. Johns, A. T., Biochem. J., 42, p. ii (1948).
- 18. Utter, M. F., and Chenoweth, M. T., Federation Proc., 8, 261 (1949).

THE EFFECTS OF TRYPTOPHAN DEFICIENCY UPON ENZYME ACTIVITY IN THE RAT*

By J. N. WILLIAMS, JR., AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, October 22, 1949)

Recently it has been observed that liver enzyme activity may be markedly affected by dietary conditions. Seifter et al. (1) have demonstrated that during complete dietary protein deprivation loss of arginase and p-amino acid oxidase from rat liver occurs more rapidly than loss of general liver protein. We (2) have observed that the level of xanthine oxidase activity in rat liver may be altered by subtle protein changes in the ration. Adult rats fed a diet completely adequate for maintenance showed a decreased liver xanthine oxidase content which was returned to normal by addition of methionine to the ration. It appeared that liver xanthine oxidase activity was a fairly sensitive index of dietary amino acid availability.

In a previous study of the effects of methionine deficiency upon liver enzyme activity, it was noted that complete deprivation of dietary methionine reduced liver xanthine oxidase activity almost completely, decreased succinic oxidase activity somewhat, and had no demonstrable effect upon liver endogenous respiration.

In the present paper the study of the effects of amino acid deficiencies upon liver enzyme activity has been extended to tryptophan. By studying the relation of individual amino acid deficiencies to liver enzyme activity, it is hoped that some light will be thrown upon amino acid, enzyme, and animal protein metabolism in general.

The enzyme systems we have chosen for study in this work are liver xanthine oxidase, succinic oxidase, cathepsin, endogenous respiration, and fatty acid oxidase. Since xanthine oxidase activity has been observed to be very sensitive to dietary protein changes, this enzyme was one of those chosen for the present study. The possibility that increased breakdown of body proteins during a tryptophan deficiency might involve a greater need for liver protease activity led us to study liver catheptic activity as a

^{*} Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and from The Robert Gould Research Foundation, Inc., Cincinnati, Ohio.

¹ Williams, J. N., Jr., Denton, A. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **72**, 386 (1949).

function of tryptophan deficiency. Since endogenous respiration of the liver may be taken as a measure of general liver metabolic rate, the effects of lack of dietary tryptophan upon this factor were studied. Both succinic oxidase and fatty acid oxidase have been shown by Schneider (3) and Kennedy and Lehninger (4), respectively, to be centered in the cell mitochondria. Therefore, deficiency studies of these enzymes might give some evidence concerning metabolism of the particulate cell matter.

EXPERIMENTAL

Adult male rats of the Holtzman strain weighing from 290 to 310 gm. were divided at random into two groups and placed in individual cages. One group was given a complete ration and the other a tryptophan-free ration for at least 2 weeks before being used in the enzyme studies. Both groups were fed and watered ad libitum throughout the feeding period. The complete ration has been described in a previous communication (2). Briefly it consisted of acid-hydrolyzed casein, plus a mixture of purified amino acids supplementing those destroyed during acid hydrolysis, 18 per cent; Salts IV (5) 4 per cent; a mixture of the known vitamins in sucrose base 2 per cent; corn oil 5 per cent; and sucrose to make 100 per cent. The tryptophan-free ration was the same as that described, except that DL-tryptophan was omitted from the mixture of amino acids. The acidhydrolyzed casein was shown by microbiological assay to be devoid of tryptophan. During the feeding period records were kept of amounts of food consumed by each animal. After the tryptophan-deficient animals had lost approximately 25 per cent of their body weight, both groups of animals were sacrificed for the enzyme determinations. At the time of sacrifice the control animals weighed 330 \pm 10 gm., while the tryptophandeficient rats weighed 230 \pm 5 gm.²

The animals were stunned by a blow on the head and decapitated. The livers were removed and chilled in cracked ice. Aliquots of the chilled livers were weighed, mixed with known volumes of cold buffer or water (depending upon the enzyme system to be studied), and homogenized. Aliquots of the homogenates were removed for total nitrogen, dry weight, and non-protein nitrogen determinations. Non-protein nitrogen was measured by precipitating the liver proteins in 10 per cent trichloroacetic acid, filtering, and determining nitrogen in aliquots of the filtrate. Difference in total nitrogen and non-protein nitrogen gave homogenate protein nitrogen, which, when multiplied by 6.25, gave liver protein per ml. of homogenate. Approximately 0.1 gm. portions of the unhomogenized livers were analyzed for total nitrogen both to serve as a check on the accuracy of preparing the homogenates and to give a better comparison of total liver

² Standard error of the mean.

nitrogen between animals. All nitrogen determinations were carried out in duplicate by a semimicro-Kjeldahl procedure.

Xanthine oxidase activity was determined according to the method of Axelrod and Elvehjem (6). Succinic oxidase activity was measured by the manometric method of Schneider and Potter (7). Catheptic activity was determined according to a modification of Anson's method, as outlined by Miller (8). Oxygen uptake during the first 10 minutes before the xanthine substrate was added in the xanthine oxidase determinations was taken as a measure of endogenous respiration of the livers. Fatty acid oxidase

				Enzyme ac	tivity*	
Enzyme determined	Ration	No. of animals	Based on liver protein		Based on liver dry weight	Based on liver wet weight
Xanthine oxidase	Complete	7	1.1	± 0.2†	0.50	0.141
	Tryptophan-free	6	0.49	± 0.04	0.27	0.074
Succinic oxidase	Complete	10	109	\pm 5	51	2.50
	Tryptophan-free	7	87	± 3	47	2.19
Cathepsin	Complete	8	98	± 4	53	15
	Tryptophan-free	8	99	± 5	52	15
Endogenous respiration	Complete	7	8.8	± 1.0	5.6	1.37
	Tryptophan-free	6	3.5	± 0.4	1.9	0.52
Fatty acid oxidase	Complete	4	16.4	± 0.9	11.2	
	Tryptophan-free	4	16.0	± 1.0	11.2	

Table I

Effects of Tryptophan Deficiency on Enzyme Activity in Rat Liver

activity was assayed by a slight modification of Lehninger's method (9). All enzyme activities were determined at 30°.

RESULTS AND DISCUSSION

It was observed during the feeding period that both groups of animals consumed approximately the same amount of ration. Therefore, any effects of protein starvation due to decreased food intake after the onset of the amino acid deficiency were negligible. A true tryptophan deficiency was present in the animals of that group, as evidenced by rapid loss of weight and extreme nervousness of the animals.

Results of the enzyme determinations are presented in Table I. Standard errors of the means are reported only for enzyme activity based on liver protein, although approximately the same relative deviations hold for

^{*} For individual enzyme units see the text.

t Standard error of the mean.

the other results. The enzyme activities are reported in terms of liver protein, liver dry weight, and liver wet weight, since dilution of the liver by lipide or glycogen might cause erroneous conclusions to be drawn from the enzyme results. Enzyme activity in terms of liver protein negates those effects.

Xanthine oxidase activity is expressed in microliters of oxygen uptake per hour per gm. of liver protein, dry liver, and fresh liver. Succinic oxidase activity is reported as microliters of oxygen uptake per hour per mg. of liver protein, dry liver, and fresh liver. Units of cathepsin activity are defined as gm. of hemoglobin nitrogen made soluble per 3 hours per gm. of liver protein, dry liver, and fresh liver. Endogenous respiration is expressed as microliters of oxygen uptake per hour per mg. of liver protein, dry liver, and fresh liver. Fatty acid oxidase is measured with octanoate as substrate in terms of microliters of oxygen uptake per hour per mg. of liver protein and dry liver. Calculation on the basis of fresh weight of liver could not be made in this case, since only the centrifugable portion of the homogenates was employed.

From Table I it can be seen that liver xanthine oxidase activity again appeared to be highly labile under adverse dietary protein conditions. A tryptophan deficiency, however, did not reduce activity of this enzyme to zero as in a methionine deficiency.¹ Succinic oxidase activity was also decreased faster than other liver protein in a tryptophan deficiency, although disappearance of activity of this enzyme was not nearly as fast as that of xanthine oxidase. It is perhaps significant that the ratio of enzyme activities of the two groups of animals when based on liver protein is different from the ratio when the activities are based upon liver, dry weight or fresh weight. The livers of the tryptophan-deficient animals were not noticeably infiltrated with fat as in a methionine deficiency. From Table II it can also be seen that in the tryptophan deficiency no detectable decrease in liver protein per gm. of liver occurred.

Catheptic activity of livers of the tryptophan-deficient animals appeared to be conserved during the deficiency period. Miller (8) observed that during acute inanition in the rat activity of this enzyme is retained more tenaciously than other liver protein. The reasons for conservation of this enzyme are not clear. However, the fact that in our results catheptic activity is lost at the same rate as general liver protein is significant.

It has frequently been reported from this laboratory that dietary conditions appear to have little effect upon liver endogenous respiration. In the present results, however, there was a sharp decrease in endogenous respiration in a tryptophan deficiency. This may be due to the possible conversion of the tryptophan to coenzymes I and II via nicotinic acid. This appears doubtful, however, since both groups of

animals received the same adequate level of nicotinic acid in the diet. It is possible that activities of enzymes involved in endogenous respiration are decreased in a tryptophan deficiency in a similar fashion to xanthine oxidase activity. If this is so, tryptophan must be very specific for that action, since a methionine deficiency and various vitamin deficiencies (10, 11) have little effect upon liver endogenous respiration.

The complex fatty acid oxidase system appeared to be essentially unaffected by a tryptophan deficiency. Although both fatty acid oxidase and succinic oxidase are centered in the cell mitochondria, activity of one may be retained by the liver and activity of the other lost.

In Table II are reported general characteristics of livers of animals of the two groups. Although the tryptophan-deficient animals lost weight rapidly, the protein proportion of the liver was not significantly altered. It has been reported by Seifter et al. (1) and others that during total pro-

Liver component measured	Ration	No. of animals	Component per 100 gm. liver	
			per cent	
Protein	Complete	18	$16.2 \pm 0.5*$	
	Tryptophan-free	18	16.2 ± 0.4	
Non-protein nitrogen	Complete	18	0.29 ± 0.003	
	Tryptophan-free	18	0.28 ± 0.01	
Dry weight	Complete	23	28.2 ± 0.4	
	Tryptophan-free	20	28.1 ± 0.2	

TABLE II

Effects of Truptophan Deficiency on General Liver Characteristics

tein deprivation nitrogen per gm. of liver is measurably decreased. It is probable that during a tryptophan deficiency the conditions of negative nitrogen balance are less drastic than complete protein starvation and the ratio of liver protein to liver water, glycogen, and lipide was not noticeably changed. This is upheld by the fact that the per cent of liver dry weight was unchanged by the tryptophan deficiency. It might be expected that non-protein nitrogen of the liver would be increased in the deficient animals. As the results indicate, however, this liver factor remained constant in the tryptophan deficiency.

From the results of these experiments it appears that a tryptophan deficiency in the rat is sufficiently drastic to cause a marked loss in activities of certain enzyme systems. The effects of the deficiency upon enzyme and general liver characteristics, however, appear to be different both qualitatively and quantitatively from the effects of inanition, protein depri-

^{*} Standard error of the mean.

vation, and methionine deficiency. The effects of other amino acid deficiencies upon enzyme systems are being studied by the authors at present. It is hoped that any differences in metabolic effects of the individual amino acid deficiencies will be brought out by these studies.

SUMMARY

- 1. A tryptophan deficiency in the rat reduces markedly liver xanthine oxidase activity and endogenous respiration. Succinic oxidase is also noticeably decreased. Activities of cathepsin and fatty acid oxidase are not altered by this deficiency condition.
- 2. General liver characteristics such as liver protein, total solids, and non-protein nitrogen per gm. of liver remain unchanged in a tryptophan deficiency.
- 3. Qualitative and quantitative interrelationships of a tryptophan deficiency, complete inanition, protein deprivation, and methionine deficiency in the rat to liver enzyme activity and other liver factors have been discussed.

BIBLIOGRAPHY

- Seifter, S., Harkness, D. M., Rubin, L., and Muntwyler, E., J. Biol. Chem., 176, 1371 (1948).
- 2. Williams, J. N., Jr., and Elvehjem, C. A., J. Biol. Chem., 181, 559 (1949).
- 3. Schneider, W. C., J. Biol. Chem., 165, 585 (1946).
- 4. Kennedy, E. P., and Lehninger, A. L., J. Biol. Chem., 172, 847 (1948).
- 5. Phillips, P. H., and Hart, E. B., J. Biol. Chem., 109, 657 (1935).
- 6. Axelrod, A. E., and Elvehjem, C. A., J. Biol. Chem., 140, 725 (1941).
- 7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
- 8. Miller, L. L., J. Biol. Chem., 172, 113 (1948).
- 9. Lehninger, A. L., J. Biol. Chem., 161, 437 (1945).
- 10. Stare, F. J., and Elvehjem, C. A., Am. J. Physiol., 105, 655 (1933).
- Williams, J. N., Jr., Nichol, C. A., and Elvehjem, C. A., J. Biol. Chem., 180, 689 (1949).

FREE AMINOETHYLPHOSPHORIC ESTER IN RAT ORGANS AND HUMAN TUMORS*

By JORGE AWAPARA, ALTON J. LANDUA, AND ROBERT FUERST (From The University of Texas, M.D. Anderson Hospital for Cancer Research, Houston)

(Received for publication, October 29, 1949)

In the course of an investigation on the free amino acids of rat organs by means of paper chromatography, several compounds were detected which could not be identified with any of the known amino acids. One component was always found in relatively large amounts in the spleen and in several human tumors (1). The position of this unknown component corresponded closely with aminoethylphosphoric ester in the map of spots of Dent (2). A similar or perhaps identical substance was reported by Work (3) in hydrolysates of Corynebacterium diphtheriae. She attempted to identify it by means of alkaline phosphatase, but her results were negative. No details of her procedure were given.

The results presented in this paper show that the unknown substance found in spleen and tumors is in fact aminoethylphosphoric ester. The analyses were extended to twenty-three organs of the rat and five human tumors. In every case aminoethylphosphoric ester was found in variable concentrations.

Methods

The extracts of rat organs were made according to a procedure described by Awapara (4). Tumor specimens were obtained from surgery and processed immediately after removal to avoid any possible decomposition. Chromatographic analyses were carried out with phenol and 2,4-lutidine as solvents. Quantitative determination of the fractions was made by the method of Landua and Awapara (5).

The synthesis of aminoethylphosphoric ester was carried out according to the procedure given by Plimmer and Burch (6).

EXPERIMENTAL

The presence of the unknown compound in beef spleen was first established by means of paper chromatography. It was shown that the substance from beef spleen was identical with the substance present in rat spleen and in human tumors, in so far as chromatographic behavior is concerned.

*This work was supported in part by a grant (No. INSTR 23B) from the American Cancer Society.

Partial Fractionation of Beef Spleen—About 1 kilo of fresh beef spleen was cut into small pieces, covered with water, acidified with acetic acid, and heated for a couple of hours. The broth was filtered through a large Büchner funnel. The residual pieces of tissue were homogenized in a Waring blendor with hot acidified water. The pulp was filtered and the resulting filtrate combined with the first one. The combined filtrates were evaporated on a water bath to a small volume. After cooling, the syrupy extract was treated with sufficient ethanol so that the final alcohol concentration would be 90 per cent. A heavy white yellowish precipitate was formed. The precipitate was separated by centrifugation, followed by one washing with 80 per cent ethanol. Samples of the precipitate and supernatant were examined by paper chromatography. The major portion of the unknown substance was found in the precipitate accompanied by other compounds which gave positive ninhydrin reaction.

Isolation of Unknown Fraction—Attempts were made to isolate the unknown fraction by means of specific adsorbents. Lloyd's reagent removed most of it from the solutions of the crude extract, but recoveries from the column were very low and heavily contaminated. Acid-treated alumina was also found effective in removing the unknown, but again the recoveries were low. The main contaminants in every case were aspartic and glutamic acids. Removal of the dicarboxylic acids by the Foreman method was tried and the unknown substance precipitated nearly quantitatively with the acid.

Paper chromatography itself was tried out as a method for isolating small amounts of the unknown substance. The capacity of filter paper is not very large, but sufficient material was isolated by using the following procedure: An aqueous solution of the alcoholic precipitate was made to contain 1 gm. in 10 ml. The clear solution was applied along a line on one edge of a large sheet of filter paper, Whatman No. 4, in such a way as to form a thin line. After drying, the application was repeated. In this manner, about 1 ml. of solution could be applied on one sheet of paper. Ten such papers were prepared and chromatography carried out with phenol for 30 hours. A 2 cm. strip was cut from the dried chromatogram and developed with ninhydrin. With the strip as a guide, the section corresponding to glutamic acid was cut and eluted with water. The unknown overlapped with glutamic acid in the phenol run so that a second resolution became necessary. The eluates from all ten chromatograms were pooled and evaporated to a small volume in the water bath. Chromatography of this solution was carried out with 2,4-lutidine in the same manner as described above. Glutamic acid and the unknown were completely resolved by this procedure. The unknown material was eluted out of the paper in fairly pure state. No other ninhydrin-reacting substance could be found on chromatography with several different solvents.

Identification of Unknown Fraction—Aliquots of the paper eluate were hydrolyzed with 6 N hydrochloric acid and 20 per cent sodium hydroxide for 24 hours in sealed tubes. Acid hydrolysis caused no change in the chromatographic behavior of the unknown. Alkaline hydrolysis, however, brought about some changes, but no new spots were detected. Tests for phosphoric acid were carried out by the Fiske and Subbarow procedure. The alkaline hydrolysates gave a strong positive test, whereas the unhydrolyzed sample was negative. The chromatographic behavior of the unknown was not changed by oxidation with 30 per cent hydrogen perox-

Table I

Approximate Concentration of Aminoethylphosphoric Ester in Rat Organs and Human Tumors

The manualta and			100		_ c	f L	41
The results are	given in	mg.	per 100	gm.	Οī	iresn	ussue

Rat organ		Rat organ	
Liver (5)	17	Salivary gland (2)*	19
Kidney (5)	Trace	Thymus (adult) (6)*	90
Muscle (5)	20	" (young) (4)*	88
Heart (5)	20	Pituitary (35)*	45
Spleen (5)	116	Thyroid (37)*	7
Brain (5)	12	Adrenals (25)*	40
Ileum (5)	6	Pineal body (87)*	Presen
Peyer's patches (5)	27		_
Colon (5)	28	Human tumors	
Pancreas (5)	100		
Lymph nodes (5)*	105	Skin carcinoma	29
Ovary (3)	60	Breast carcinoma	58
Uterus (4)	15	Hepatoma	24
Testis (5)	29	Ovary carcinoma	37
Seminal vesicle (5)*	9	Spleen, leucemia	55
Coagulating gland (7)*	6		

The figures in parentheses represent the number of animals.

ide. With this information on hand, it seemed highly probable that the unknown fraction was indeed aminoethylphosphoric ester.

The final proof was furnished by the identification of ethanolamine in the hydrolysate. Because of the resistance of ethanolamine phosphoric ester to acid hydrolysis, it was necessary to carry out the reaction with 6 n hydrochloric acid in a sealed tube for 45 hours. At the end of this period, sufficient ethanolamine was released for identification by means of paper chromatography. Known solutions of ethanolamine were treated with hydrochloric acid in the same manner as the unknown. Chromatography of both solutions gave identical spots.

^{*} The samples were pooled

Further proof was obtained by chromatography of a mixture of the unknown substance with synthetic aminoethylphosphoric ester. In all solvents tried, a single homogeneous spot was obtained.

Incubation of the eluate with rat kidney extracts brought about complete disappearance of the aminoethylphosphoric ester.

RESULTS AND DISCUSSION

The fraction identified as aminoethylphosphoric ester in beef spleen was found to occur in nearly every organ of the rat and in several human tumors. The relative concentration in each organ is given in Table I. The values reported probably do not represent the absolute values because of the insolubility of aminoethylphosphoric ester in alcohol. However, all values are equally obtained and give an index of the relative concentration in each organ.

The results of this investigation indicate that aminoethylphosphoric ester is a widely distributed metabolite. Outhousen (7, 8) isolated the ester from several tumors but failed to detect it in various normal organs examined. Using improved methods of isolation, Colowick and Cori (9) presented evidence of the existence of aminoethylphosphoric ester in pig and rabbit intestine. Chargaff and Keston (10) determined the rate of phosphatide formation in vivo from aminoethylphosphoric ester containing the radioactive isotope of phosphorus. Their results suggested that tumors belong to the most active tissues with regard to the formation of phosphatides from aminoethylphosphoric ester.

SUMMARY

- 1. Aminoethylphosphoric ester was identified in twenty-three organs of the rat and several human tumors. This substance was isolated from beef spleen by means of paper chromatography and identified by the same means.
- 2. Approximate values for the concentration of aminoethylphosphoric ester in organs of the rat and in tumors are given.

BIBLIOGRAPHY

- 1. Awapara, J., Landua, A. J., and Fuerst, R., Biochim. et. biophys. acta, in press.
- 2. Dent, C. E., Biochem. J., 43, 169 (1948).
- 3. Work, E., Biochem. et biophys. acta, 3, 401 (1949).
- 4. Awapara, J., Arch. Biochem., 19, 172 (1948).
- 5. Landua, A. J., and Awapara, J., Science, 109, 385 (1949).
- 6. Plimmer, R. H. A., and Burch, W. J. N., Biochem. J., 31, 398 (1937).
- 7. Outhousen, E. L., Biochem. J., 30, 197 (1936).
- 8. Outhousen, E. L., Biochem. J., 31, 1459 (1937).
- 9. Colowick, S., and Cori, C. F., Proc. Soc. Exp. Biol. and Med., 40, 586 (1939).
- 10. Chargaff, E., and Keston, A. S., J. Biol. Chem., 134, 515 (1940).

METABOLIC STUDIES OF AZOTOBACTER AGILIS BY THE USE OF A MUTANT DEFICIENT IN PYRUVIC OXIDASE*

By J. L. KARLSSON

(From the Division of Plant Nutrition, University of California, Berkeley)

(Received for publication, August 22, 1949)

An account has been given elsewhere of the successful isolation of physiological x-ray mutants of Azotobacter agilis with genetic blocks in the substrate oxidation mechanisms (1). The present paper deals in greater detail with a mutant strain, designated A13, that was isolated as a variant unable to grow on glucose, and gives the methods used to characterize the particular deficiency responsible for this alteration.

Materials and Methods

The source of the organism, as well as the methods of culture and the media, has been described in previous publications (1, 2). Strain A4.4 is the original organism and strain A13 is a mutant isolated from an x-ray-treated culture.

The methods for the respiration experiments with cell suspensions have also been published (2), except for the slight modification for carbon dioxide determination. This was accomplished by setting up a series of identical Warburg cups without alkali in the center well and releasing the carbon dioxide from the neutral medium with acid at different stages. The oxygen consumption was determined at the same time in a cup with KOH and the amount of carbon dioxide computed from the difference. In the particular case in question the rate of oxygen uptake was shown to be independent of the presence of CO_2 .

Pyruvic acid was estimated by Lu's colorimetric method as modified by Friedemann and Haugen, with toluene as the extraction solvent (3). The same method, with ethyl acetate instead of toluene, was employed for total keto acid determinations. Since the light absorption curves for the 2,4-dinitrophenylhydrazones of pyruvate and oxalacetate are identical (3) and these two acids were the only keto acids present, this estimation coupled with the pyruvate determination could be used to determine oxalacetate.

cis-Aconitic acid was synthesized from trans-aconitic acid by the method of Malachowski and Maslowski (4).

* The material presented in this paper is taken from a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of California in June, 1947.

Results

It has already been reported (1) that strain A13 is completely unable to develop on the following compounds which are utilized for growth by the parent strain: pyruvate, lactate, malate, fumarate, succinate, α -ketoglutarate, cis- and trans-aconitate, tartrate, glucose, fructose, and gluconate. Growth of the mutant is supported only by ethyl alcohol, acetic acid, and malonic acid, and on these three substrates, it is indistinguishable from that of the original strain. Neither organism will grow on mannitol,

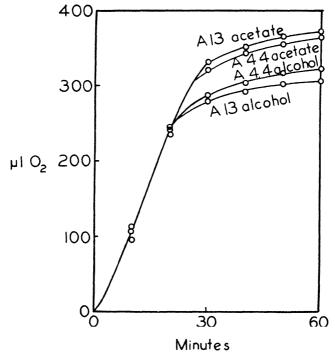


Fig. 1. Respiration curves for strains A4.4 and A13 on alcohol and acetate. Theoretical oxygen uptake for complete oxidation, $448 \mu l$. of O_2 .

galactose, xylose, p-arabinose, 2-ketogluconate, glycerol, citrate, diacetyl, propionate, oxalate, formate, glutamate, aspartate, alanine, or glycine. Both are able to adapt themselves to butyrate, apparently by a mutation mechanism, since only a few colonies develop. This change does not affect utilization of other substrates.

The two strains exhibit identical behavior, not only in regard to growth but also as concerns respiratory rates, when tested on ethyl alcohol or acetate. This is illustrated in Fig. 1. Certain compounds which sup-

port growth of strain A4.4, but not of strain A13, are still oxidized by the latter, as shown in Fig. 3. For comparison the respiratory curves for the parent strain on the same compounds are given in Fig. 2. The same quantity of cells was used in the experiments shown in Figs. 2 and 3.

It will be noted on the respiration curves for strain A13 metabolizing the 4-carbon dicarboxylic acids that the rate is always slow and that a distinct break occurs when less than the theoretical amount of oxygen for complete oxidation has been consumed (Fig. 3). The same is true for α -ketoglutarate, which has a much longer adaptation period. Pyruvate is

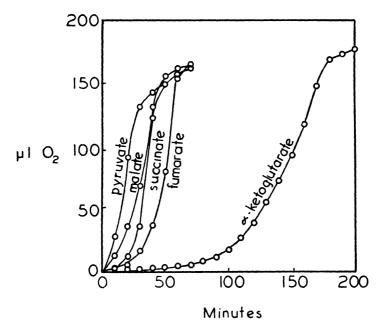


Fig. 2. Respiration curves for strain A4.4, grown on alcohol. Theoretical oxygen uptake for all substrates, $224 \mu l.$ of O_2 .

the only slowly oxidized compound which is used at a constant rate, and the oxygen consumption for the other substances, after the break, levels off to that same rate. The oxygen taken up before the inflection corresponds to the amount required for oxidation to the pyruvate stage. The breaks occur at different times, and the total amount of oxygen consumed at these points is the sum of that used to convert the substrate to pyruvate and that subsequently used up in its oxidation. It should require 5 μ M or 112 μ L of oxygen to convert 5 μ M of succinate to the oxidation state of pyruvate, while fumarate and malate require only half that amount. If we allow for experimental error, it is evident that the distances from the

pyruvate curve to the other curves on Fig. 3, after the breaks have occurred, are in this order of magnitude. A comparison of the CO₂ curve with the oxygen curve of malate (Fig. 4) shows that very little carbon has been split off before the break, suggesting that oxalacetate rather than pyruvate accumulates as an intermediate product during the oxidation. This is confirmed by the data in Table I which show the formation of keto

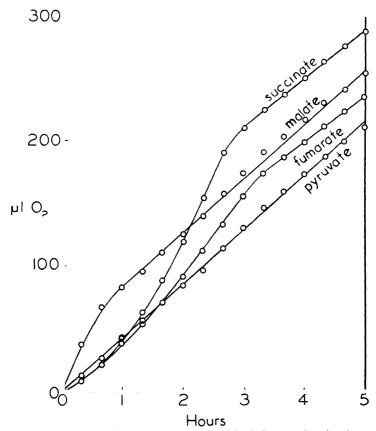


Fig. 3. Respiration of strain A13, grown on alcohol. $5 \,\mu\mathrm{m}$ of each substrate

acids during the oxidation of malate. The difference between the total keto acids and pyruvate is taken as oxalacetate. Isolation of the keto acid by the method of Clift and Cook (5) was unsuccessful, a syrup being obtained instead of crystals. But the light absorption curve for this derivative was identical with that of oxalacetate. Furthermore, the compound was decomposed to form the theoretical amount of carbon dioxide by the aniline citrate method. This leaves little doubt that it is actually

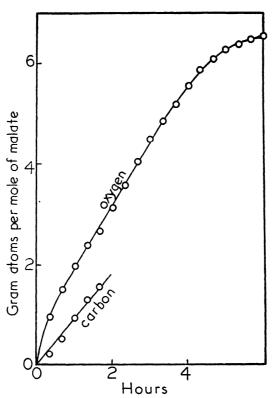


Fig. 4. Oxygen uptake and carbon dioxide production by strain Al3 on malate. Theory for complete oxidation, 6 gm. atoms of oxygen and 4 gm. atoms of carbon per mole of malate.

Table I Keto Acids Formation during Malate Oxidation by Strain A13 Initial malate, $5.00~\mu M$. The break on the O_2 uptake curve occurred at 20 minutes (see Fig. 4).

Time	Oxalacetate	Pyruvate	Total
min.	μ Μ	μМ	μМ
0			0.04
20	3.89	0.75	4.64
60	3.04	1.96	5.0 0

oxalacetate. In Table I the value for total keto acids is somewhat higher than that expected after 1 hour, probably owing to an experimental error. But in any event, the evidence is fairly conclusive that the mutant strain has a metabolic block in the oxidation of pyruvate and oxalacetate.

Further experiments were undertaken to find out whether this disturbance was the only metabolic effect of the mutation. Certain compounds such as glucose can be decomposed by the parent strain, but not by the mutant, within the time limit of these experiments. All these substrates have one thing in common: they always require a long adaptation period before they are attacked by alcohol-grown cells. Since the mutant shows in general a slower rate of adaptation than the normal strain when exposed to compounds above the block, it would not be expected to be able to adapt itself within the time limit, since it is not entirely stable and reverts to the parent type on exposure to the new substrate for a day or longer.

The rates of attack on compounds which could be conveniently studied were compared in the following manner. Strains A4.4 and A13 were grown in 0.2 per cent alcohol. After growth had ceased due to depletion of the alcohol, 1 per cent succinate was added, and incubation was con-

Table II Respiratory Rates for Succinate-Adapted Cells, Expressed As QO2 (N) \times 10 2

	Observed rates		Computed initial rates		
	Strain A4.4	Strain A13	Strain A4.4	Strain A13	
Succinate	10.7	2.0	1.5	0.9	
Fumarate	7.6	0.9	1.3	0.7	
Malate	11.6	10.8	1.9	10.6	
Pyruvate	8.2	0.2	1.6	0.04	
Acetate	15.8	16.3	4.0	4.1	
-					

tinued on a shaker overnight. The cells, which should have become adapted to succinate and its breakdown products, were harvested and used for a $Q_{\rm o_2}$ (N) determination. The rate during the second 10 minute interval following substrate addition was used in the calculation.

The resulting respiratory rates are shown in the first two columns of Table II. Since these are over-all rates for reactions, some of which lead to accumulation of an intermediate, their significance is not immediately apparent. The rate of oxygen uptake with a given substrate by two organisms may differ greatly because of differences in the completeness of oxidation, even though the initial rate is the same. To overcome this difficulty, the rate at which the first step proceeds in the oxidation of each substrate was computed. Since there is no evidence of breaks on the oxygen curves for succinate, fumarate, and malate obtained with strain A4.4, the rate for the initial step should be the over-all rate divided by the number of oxygen atoms required for complete oxidation. The same is

true for the oxidation of pyruvate and acetate by both strains. For strain A13, when a break occurs at the pyruvate stage, the initial rates for succinate, fumarate, and malate are the observed rates less the rate for pyruvate divided by the number of oxygen atoms required before the pyruvate stage. As an example, the observed rate for strain A13 on succinate is 2.0 ml. per hour per mg. of cell N. The rate for the oxidation to the pyruvate stage is this less the observed rate for pyruvate (0.2), or 1.8. 2 oxygen atoms are required to convert succinate to pyruvate; hence the rate of the initial oxidation is 0.9. The figures obtained by this calculation are shown in the last two columns of Table II. Pyruvate is the only substrate oxidized at a greatly reduced rate by strain A13. The reason why the initial rates for succinate and fumarate are also somewhat lower might be that adaptation in the mutant was less complete than in the normal strain, so that succinic dehydrogenase and fumarase were not built up to their highest levels. The surprisingly high oxidation rate of strain A13 for malate, which undergoes just one oxidative step before the break, is hard to reconcile with the rate for this compound in the normal strain. It does not seem likely that the mutant is actually able to oxidize malate at a faster rate. More probably the rate is close to the maximum possible for O₂ utilization on this substrate, and in the absence of competition for oxygen by lower products in the mutant, more is available for malate oxidation. Whatever the reason for this high rate may be, it is evident that pyruvate is the only substrate in this group of compounds whose oxidation is materially slower in the mutant than in the parent The initial rates of attack on succinate and fumarate are similar in both strains.

Although substrates other than acetate, ethyl alcohol, and malonate do not support the growth of the mutant, it was of interest to find out whether the energy from the oxidation of such substrates was utilizable for growth of the organism when acetate was also provided. The acetate is oxidized in preference to the other substrate, but since a certain amount of the latter is also decomposed, growth would be expected to be somewhat heavier than on acetate alone. Media containing acetate and succinate were inoculated with cells already adapted to oxidize succinate. The amount of growth was determined after 24 hours. As shown in Table III, a definite increase in growth was caused by the succinate. Therefore the oxidation of succinate must in some way be useful to the organism, even though succinate cannot support growth by itself.

A series of experiments was done in an attempt to prove that pyruvate is oxidized by way of acetate in the normal strain. From what is known of the transformation of pyruvate in other microorganisms, it seems very likely that this is the main metabolic path of pyruvate in Azotobacter, and

this interpretation is supported by adaptation experiments which have previously been described (2). A test for anaerobic decarboxylation of pyruvate by a manometric method was negative, showing that the initial attack on this substrate is indeed dependent on oxygen. An indication of a common pathway for pyruvate and acetate was obtained by a determination of the respiratory rate for each compound separately and for both combined. Succinate was added in one Warburg vessel to show that the maximum possible rate of oxidation had not been reached. Table IV shows that the rate on pyruvate and acetate together is no higher than that on acetate alone, as would be expected if the compounds are competing for the same enzymes. The significance of this experiment may be ques-

Table III

Influence of Succenate on Growth of Strain A13 on Acetate

Substrate, 0 02 M	Turbidity at 24 hrs	N₂ fixed	
	2 - log G	mg.	
Acetate	0 13	0 92	
Succinate	0 00	0 00	
Acetate + succinate	0 16	1 36	

Table IV

Separate and Combined Respiratory Rates for Lactate-Grown Strain A4.4, Adapted to Succinate

Substrate	$Q_{\mathbf{O_3}}$ (N) \times 10 ⁻²
Pyruvate	14
Acetate	16
Pyruvate + acetate	16
Succinate + "	21

tioned, since the possibility that the rate of O_2 uptake may be limiting is not definitely excluded, because succinate does stimulate the rate of acetate oxidation under certain conditions.

An attempt was made to prove directly that pyruvate is oxidized via acetate by an isotope dilution method. Both pyruvate and C¹⁴-labeled acetate were added to a culture. Unfortunately the acetate was decomposed preferentially when both substrates were present; thus the results were inconclusive. The theory that pyruvate is oxidized via acetate has therefore not been proved directly, but at least no evidence has been found against it.

Finally some experiments were carried out in an attempt to show that malonate is decomposed via acetate. According to Lineweaver (6) the

first step is a decarboxylation to form acetate. However, his data are entirely inconclusive for two reasons: first, he used cells which were not adapted to malonate; second, the amount of carbon dioxide which he found to be split off before oxygen consumption started was too small to be measured by the Warburg technique. Obviously carbon dioxide liberation unassociated with oxygen uptake could not be determined by malonate-adapted cells under aerobic conditions, since such cells oxidize acetate readily. A manometric experiment was therefore done with malonategrown cells suspended in a malonate medium under anaerobic conditions. Only a slight increase in pressure, amounting to approximately 10 per cent of the theoretical for complete decarboxylation to acetate, was observed and this soon ceased. Apparently the initial attack on malonate is dependent on oxygen. However, the rôle of oxygen in malonate decarboxylation is not clear. Oxygen might cause an oxidation of the malonate before it is decarboxylated or it might bring about an activation of the malonate, for example, by a phosphorylative mechanism.

DISCUSSION

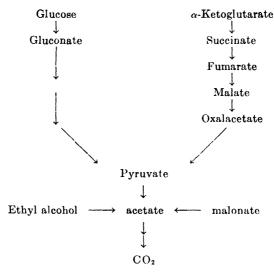
The experiments in this paper have shown that there is no reason to suspect anything abnormal in the metabolism of strain A13 except the rate of breakdown of pyruvate. From Table II it is evident that succinate, fumarate, and malate can be attacked at a rate comparable with that of the normal strain if adaptation to the first two substances is permitted to take place. This adaptation is also required by the normal strain. The over-all rate for these compounds is always slower in the mutant, as is to be expected, since the reaction is practically arrested when oxalacetate or pyruvate is reached.

All the anomalies of strain A13 can be accounted for if it is assumed that this organism has a metabolic block in the conversion of pyruvate to acetate. To explain the growth disturbances it has to be further postulated that acetate or some similar oxidation product is indispensable for growth of A. agilis and must be supplied either as such or in the form of substances which can be oxidized to acetate. Any compound which cannot be converted to acetate is then unable to support growth. This assumption is not an unreasonable one, since independent evidence has been obtained for the constant formation of acetate from other substrates in the normal strain (2), and since the experiments in this paper have shown that the mutant strain can oxidize higher substrates and obtain energy from them. It must be noted that acetate is required in large quantities, since most of it is rapidly decomposed to carbon dioxide and water. In the case of strain A13, acetate or an acetate substitute can still be formed from alcohol and malonate.

The fact that pyruvate is still attacked at a slow rate by the mutant

strain has to be accounted for. The rate is only one-fortieth of the normal, as shown in Table II. No growth accompanies this oxidation; thus it is certainly not leading to the same products as oxidation of pyruvate by the normal strain. If acetate were formed, the organism would be expected to grow, since a mixture of pyruvate and acetate permits growth. The theory is therefore suggested that pyruvate is decomposed in the mutant by a slow, secondary mechanism which does not lead to acetate.

Certain conclusions concerning the pathways of substrate oxidation by A. agilis can be drawn from the behavior of the mutant strain. seem reasonable to assume that any compound that supports growth of the normal strain, but not of the mutant, is normally metabolized by way of pyruvate. A list of these substances has already been given. Evidence has been presented in a previous publication for the existence of a chain of reactions starting with α -ketoglutarate and going through succinate, fumarate, malate, oxalacetate, and pyruvate to acetate (2). On the other hand, unpublished experiments have shown that glucose-grown cells are unable to oxidize α -ketoglutarate, succinate, or fumarate until time has been permitted for adaptation to take place, while pyruvate is readily attacked without adaptation. This indicates that the hexose sugars are oxidized to pyruvate by a pathway not involving α -ketoglutarate. The actual mechanism leading from glucose to pyruvate is not known, but gluconic acid is a suspected intermediate, because growth on glucose leads to adaptation to gluconate, while the reverse is not the case. This was shown by Harris (7) and confirmed by me. On the basis of these considerations the accompanying scheme is suggested for the metabolic path-



ways in A. agilis. Acetate is oxidized to carbon dioxide by a completely obscure mechanism which does not seem to be related to the tricarboxylic acid cycle (2).

Another observation worthy of further mention is that adaptation rates are slow for the mutant when exposed to the substrates above the block, indicating that acetate or other oxidation products of pyruvate may be involved in enzyme synthesis. This prolongation of adaptation is not due to the absence of growth, since adaptation normally takes only a few minutes for certain substrates and must therefore be independent of growth.

Finally, it should be pointed out that the use of mutants for the study of catabolic reactions may be more limited than was thought at the outset. This is due to the adaptation difficulties. For instance, there was in the beginning no reason to suspect that strain A13 could not be used to demonstrate directly that glucose or any other complex substrate is metabolized via pyruvate. However, the strain can only be grown on acetate, alcohol, or malonate, and is therefore unadapted to other substrates. Adaptation to attack glucose at a sufficiently high rate could not be accomplished within the time limit since the mutant is not entirely stable. It had been expected that any mutant strain with a block in some major metabolic path might require two substrates for growth, one to supply the compounds above the block, the other the ones below. With strain A13 this was not true, possibly because the synthetic path differs from the oxidative one. But this would not always be expected to be the case, and a strain requiring two substrates for growth would naturally be adapted to both. Therefore, although the method has its limitations, it no doubt might prove to be a very important tool to eliminate one enzyme at a time from metabolic systems and cause accumulation of intermediate oxidation products.

SUMMARY

A mutant strain of Azotobacter agilis, characterized by inability to grow on any of the ordinary substrates except acetate, ethyl alcohol, and malonate, has been shown to be able to oxidize several more complex compounds to oxalacetate or pyruvate which accumulate in the medium. The energy derived from the oxidation of higher substrates is available to the organism, but growth is apparently impossible unless acetate or a similar compound either is supplied as such or can be formed during growth. All the abnormalities of the mutant can be explained by assuming that it is deficient with respect to an enzyme system catalyzing the oxidation of pyruvate to acetate. A scheme of oxidative pathways in A. agilis is suggested on the basis of previous and present evidence.

The author wishes to thank Dr. H. A. Barker for his valuable suggestions and constructive criticism throughout this work, as well as for assistance in preparing the manuscript.

BIBLIOGRAPHY

- 1. Karlsson, J. L., and Barker, H. A., J. Bact., 56, 671 (1948).
- 2. Karlsson, J. L., and Barker, H. A., J. Biol. Chem., 175, 913 (1948).
- 3. Friedemann, T. E., and Haugen, G. E., J. Biol. Chem., 147, 415 (1943).
- 4. Malachowski, R., and Maslowski, M., Ber. chem. Ges., 61 B, 2521 (1928).
- 5. Clift, F. P., and Cook, R. P., Biochem. J., 26, 1800 (1932).
- 6. Lineweaver, H., J. Biol. Chem., 99, 575 (1933).
- 7. Harris, J. O., J. Biol. Chem., 162, 11 (1946).

AN IMPROVED MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF CYSTINE IN HUMAN URINE WITH LEUCONOSTOC MESENTEROIDES P-60*

By MERRILL N. CAMIEN AND MAX S. DUNN

(From the Chemical Laboratory, University of California, Los Angeles)

(Received for publication, November 2, 1949)

Investigations concerning the urinary excretion of cystine by normal subjects and by individuals with cystinuria, liver cirrhosis, diabetes, tuberculosis, and other pathological conditions have been reported previously from this laboratory (2-6). In continuing these studies, modification of the cystine assay method seemed desirable to minimize destruction of cystine which may occur in the medium as a consequence of the Maillard reaction (7-10). The authors' investigations led to the development of a microbiological procedure by which apparent browning of the medium was entirely eliminated. The response of *Leuconostoc mesenteroides* P-60 to L-cystine under the new conditions was approximately 10 times as sensitive as that obtained by the method employed previously in the authors' laboratory (2). Although closely agreeing cystine values for acid-hydrolyzed urine were obtained by the two procedures, those for untreated urine were much lower when determined under the improved conditions.

The objectives of the present report are (a) to describe the improved assay method as applied to the determination of cystine in urine and (b) to discuss the wide variation in values obtained under different assay conditions for cystine in unhydrolyzed urine.

EXPERIMENTAL

Both acid-hydrolyzed¹ and untreated urine samples were employed. Neutralized samples diluted 1:10 to 1:40 and standard L-cystine solutions were added to 13×100 mm. test-tubes in the manner described previously (12). Five dosage levels for each urine sample and fifteen standard L-cystine levels (0 to 1.4 γ per tube) were run in triplicate in each experiment. The Brewer automatic pipette was employed for each pipetting operation throughout the experiments.

^{*} Paper 65. For Paper 64, see Camien and Dunn (1). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council. The authors are indebted to Katharine Remington Dunn, Samuel Eiduson, and Gene Molene for technical assistance.

¹ The hydrolysis procedure was essentially as described previously (11).

The basal medium was prepared as indicated in Table I. The amino acids of the medium were the same as in Medium D of Paper XVIII (14), except that cystine and hydroxyproline were omitted and the final con-

Table I
Composition of Cystine Assay Medium

Mixture A		Mixture B	
	mg.		mg.
DL-Alanine	8181	Adenine sulfate · 2H ₂ O	41.4
L-Arginine · IICl	327	Guanine · HCl · H ₂ O	39.0
L-Asparagine	1636	Uracil	36.0
L-Glutamic acid	614	Xanthine	36.0
Glycine	409	Sodium acetate	36,000
L-Histidine · HCl · H2O	82	Ammonium chloride	12,000
DL-Isoleucine	614	KH ₂ PO ₄	1,500
L-Leucine	307	K₂HPO₄	1,500
DL-Lysine·HCl	654	MgSO ₄ ·7H ₂ O	600
DL-Methionine	164	FeSO ₄ ·7H ₂ O	30
DL-Norleucine	409	MnSO ₄ ·4H ₂ O	30
DL-Norvaline	409	NaCl	1,050
DL-Phenylalanine	246	Thiamine · HCl	3.0
L-Proline	102	Pyridoxine	4.8
DL Serine	327	Pyridoxamine · 2HCl	0.30
DL-Threonine	1841	Pyridoxal·HCl	0.30
L-Tryptophan	41	Ca dl-pantothenate	6.0
L-Tyrosine	123	Riboflavin	6.0
DL-Valine	614	Nicotinic acid	6.0
		Biotin	0.015
		p-Aminobenzoic acid	0.30
		Folic acid	0.015
		Choline chloride	30.0
		Inositol	75.0

Amounts given are for 1000 assay tubes, each containing a final total of 3 ml. of solution. Mixture A and Mixture B were dehydrated and treated in a ball mill separately by the method of Camien and Dunn (13). Neither mixture was noticeably hygroscopic or harmed by prolonged storage in amberware bottles. 1.71 gm. of Mixture A and 5.16 gm. of Mixture B were dissolved in distilled water and diluted to 100 ml. to prepare medium for 100 assay tubes. 1 ml. of the solution (pH 6.32) was added to 1 ml. of diluted urine or L-cystine sample in each assay tube. The tubes were covered, sterilized in flowing steam at 100° for 30 minutes, and cooled. 1 ml. of sterile 6 per cent glucose solution was then added aseptically to each tube.

centration of the other amino acids was increased about 40 per cent. The other constituents of the medium were those given in Table I of Paper 29 (15), except that folic acid was substituted for the folic acid concentrate. The dry mixtures (Table I) were dissolved in distilled

Cystine in test mixture per tube	Titration volume*	Cystine found per tube	Cystine recovered	Deviation from mean value
γ	ml.	γ	per cent	per cent
0.188	5.71	0.182	96.7	2.1
0.375	7.08	0.372	99.1	0.3
0.563	8.08	0.546	97.1	1.7
0.750	9.28	0.768	102.3	3.5
0.938	10.00	0.930	99.2	0.4
Avorago	and the second s		08.0	1 8

TABLE II

Recovery of Cystine from Amino Acid Test Mixture

The mixture contained 2.4 per cent L-cystine in addition to the following percentages: DL-alanine 6.5, L-aspartic acid 9.7, L-arginine·HCl 3.9, L-glutamic acid 6.5, glycine 4.8, L-histidine·HCl·H₂O 4.4, DL-isoleucine 6.5, L-leucine 4.8, L-lysine·HCl 6.1, DL-methionine 3.2, DL-phenylalanine 4.8, L-proline 4.8, DL-serine 13.0 DL-threonine 9.7, L-tyrosine 2.4, and DL-valine 6.5. Three additional assays of the given mixture yielded recoveries of 100.2, 98.0, and 103.4 per cent with mean deviations from the means of 1.5, 0.8, and 3.1 per cent, respectively.

* Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.

TABLE III
Assay of Cystine in Untreated Urine

Original method

amprovou anotatou			Urine added	Autoclaved 20 min.		Autoclaved 40 min.	
Urine added per tube*	Titration volume†	Cystine found‡	per tube*	Titration volume†	Cystine found‡	Titration volume†	Cystine found:
ml.	ml.	mg.§	ml.	ml.	mg.	ml.	mg.
0.005	5.42	43.8	0.02	2.93	92.0	2.79	110
0.010	6.54	42.1	0.04	4.15	93.4	3.69	105
0.015	7.50	41.5	0.06	5.11	87.7	4.50	102
0.020	8.31	40.9	0.08	6.19	96.2	5.09	102
0.025	8.82	38.7	0.10	6.70	90.6	5.53	101
Average		. 41.4			92.0		104

- * Amounts calculated in terms of undiluted sample.
- † Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.
- ‡ Calculated in terms of excretion per 24 hours.
- § The downward "drift" of values which may be noted in this assay was not commonly observed with the improved method. The average value, 42.0, was found with two additional determinations (improved procedure) for the same sample.

water² as required and 1 ml. of the solution (pH 6.32) was added to 1 ml. of diluted urine or L-cystine sample in each test-tube. The tubes were covered, sterilized in flowing steam at 100° for 30 minutes, and cooled. 1 ml. of sterile 6 per cent glucose was then added aseptically to each tube. The tubes were autoclaved after adding the glucose in some of the experiments to obtain (for comparison) cystine assay conditions approximating those employed previously in this laboratory (2).

The methods for maintaining the L. mesenteroides P-60 culture, preparing the inoculum suspension, and inoculating the tubes have been described previously (12). It was found convenient in many of the experiments,

improved method			Original method					
			- Urine added	Autoclaved 20 min.		Autoclaved 40 min.		
Urine added per tube*	Titration volume†	Cystine found‡	per tube*	Titration volume†	Cystine found‡	Titration volume†	Cystine found‡	
ml.	ml.	mg.	ml.	ml.	mg.	ml.	mg.	
0.005			0.02	2.70	77.1	2.50	89.8	
0.010	6.08	76.5	0.04	3.61	78.6	3.09	80.3	
0.015	7.21	70.8	0.06	4.39	74.0	3.62	75.6	
0.020	8.28	70.2	0.08	5.11	70.9	3.97	66.2	
0.025	9.40	72.1	0.10	5.52	66.3	4.28	61.4	
Arrorogo		79 4			73 /		74.7	

TABLE IV
Assau of Custine in Hudroluzed Urine

however, to add inoculum suspension to the sterile 6 per cent glucose solution until the latter was barely turbid. Inoculation and addition of the glucose solution were then accomplished in a single pipetting operation.

The inoculated tubes were incubated for 72 hours at 35° and then steamed for 20 minutes at 100° before titrating as described previously (12).

The experimental results are given in Figs. 1 and 2 and Tables II to IV. Samples (prepared by Mr. I. Porush in the authors' laboratory) of precystine and p-cystine ($[\alpha]_p^{25} = +209^\circ$ in 1.003 n HCl) had activities of 49.2 and 1.5 per cent, respectively, compared to analytically pure recystine, as determined by the authors' improved procedure.

^{*} Amounts calculated in terms of undiluted sample.

[†] Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.

[‡] Calculated in terms of excretion per 24 hours.

² The small amount of insoluble residue was removed by filtration or decantation.

DISCUSSION

L. mesenteroides P-60 was selected for the determination of cystine in urine, since it has been found to be more specific in its requirement for this amino acid than Lactobacillus arabinosus 17-5, Lactobacillus fermenti 36, Lactobacillus casei, Lactobacillus pentosus 124-2, Lactobacillus delbrueckii LD-3, L. casei (delbrueckii) LD-5, and Streptococcus faecalis R (10, 16).

Considerable evidence has appeared in recent literature (7-10), indicating that the Maillard reaction in microbiological assay media is accompanied by considerable destruction of nutrients, especially cystine. Although it has been suggested that separate sterilization of the glucose (7, 9) or substitution of sucrose for glucose (8, 9) will eliminate apparent browning in the medium, it does not appear that cystine in urine has previously been determined under conditions which minimize browning and cystine destruction.

Browning of the medium was avoided in the present experiments by separate sterilization of the glucose.8 Under these conditions the amounts of cystine found in untreated 24 hour urine samples from five normal male subjects averaged⁴ 48.8, 42.0, 34.8, 63.2, and 41.2 mg. respectively. The same samples yielded 64.9, 63.5, 59.7, 89.4, and 51.1 mg. after hydrolvsis. That these values may be of relatively high accuracy was indicated by recoveries of cystine from an amino acid test mixture (Table II), averaging 100.1 per cent for four separate assays (98.8, 100.2, 98.0, and 103.4). The average deviation from the mean calculated for repeated assays of the same sample averaged 4.5 per cent for seventeen 24 hour samples (from normal and tuberculous subjects) in which cystine was determined on two to four separate occasions, indicating that a satisfactory degree of reproducibility may be obtained by the described procedure. Mean deviations from the means for the five dosage levels in the individual assays averaged 4.3 per cent for a total of 56 assays run in a number of experiments.

The values found for cystine in normal untreated urines (except for the individual who excreted 63.2 mg. per 24 hours) were considerably lower than those reported in the literature (2, 4, 5, 18, 19). Since the values (given in the preceding paragraph) found for normal urine after hydrolysis were approximately the same as those reported previously (4), it seemed likely that the discrepancy in values obtained for untreated samples was due to a difference in the extent to which the free cystine

³ It is not likely that sucrose could have been substituted for glucose since this disaccharide does not appear to be utilized by *L. mesenteroides* P-60 (17).

⁴ Each value is the average of two separate determinations.

⁵ Data for the tuberculous subjects are to be published elsewhere.

in the standard (and hydrolyzed samples) and the bound cystine in the untreated samples were destroyed.

The data given in Tables III and IV, obtained by determining cystine in a single urine sample before and after hydrolysis as well as under conditions enhancing and minimizing the Maillard reaction, appear to support this hypothesis. An untreated 24 hour urine sample yielded 41.4 mg. of

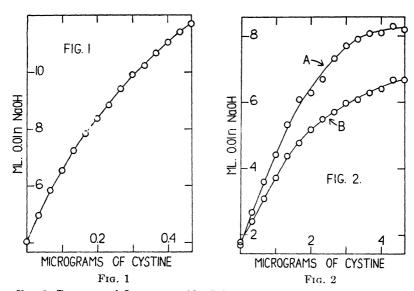


Fig. 1. Response of L. mesenteroides P-60 to L-cystine. Glucose in sterile solution was added aseptically to the assay tubes after steam sterilization of the latter (containing no glucose before sterilization) at 100° for 30 minutes. The micrograms of cystine and the ml. of 0.01 N NaOH are the amounts calculated per ml. of final solution. Each point on the curve represents the average titration from three assay tubes (13×100 mm. tubes, each containing 3 ml. of final solution).

Fig. 2. Response of L. mesenteroides P-60 to L-cystine. The assay tubes contained glucose before sterilization and were autoclaved at 15 pounds for 20 minutes (Curve A) and 40 minutes (Curve B) respectively.

cystine when assayed by the improved procedure, but 92.0 and 104 mg., respectively, were found for the same sample when the assay tubes containing glucose were sterilized in the autoclave for 20 and 40 minutes.⁶ After hydrolysis, however, this sample yielded 73 ± 2 mg. under each of the three assay conditions. It may be noted from the standard curves obtained under these varying conditions (Figs. 1 and 2) that the sensitivity of response was increased approximately 10-fold with the improved pro-

⁶ Urea or other substances present in the unhydrolyzed urines may inhibit the Maillard reaction sufficiently to explain in part these results.

cedure. Destruction of nutrients other than cystine or production of toxic substances as a consequence of the browning reaction may be indicated by the lower maxima approached by Curves A and B in Fig. 2.

SUMMARY

An improved microbiological method for the determination of cystine in urine has been described. 24 hour urine specimens from five normal male subjects yielded 35 to 63 mg. of cystine before hydrolysis and 60 to 89 mg. after hydrolysis. The analytical results appeared to be of relatively high dependability, since an average of 100.1 per cent of the acceptance in an amino acid test mixture was recovered and satisfactory agreement of results for cystine in urine samples was obtained at different dosage levels and with repeated determinations. Higher values (similar to those reported in the literature), obtained by the original procedure for cystine in untreated urine, were shown to result from unequal destruction of standard and sample cystine as a consequence of the Maillard reaction.

BIBLIOGRAPHY

- 1. Camien, M. N., and Dunn, M. S., J. Biol. Chem., 182, 119 (1950).
- 2. Frankl, W., and Dunn, M. S., Arch. Biochem., 13, 93 (1947).
- 3. Frankl, W., Martin, H., and Dunn, M. S., Arch. Biochem., 13, 103 (1947).
- Yeh, H. L., Frankl, W., Dunn, M. S., Parker, P., Hughes, B., and György, P., Am. J. Med. Sc., 214, 507 (1947).
- Dunn, M. S., Camien, M. N., Akawie, S., Malin, R. B., Eiduson, S., Getz, H. R., and Dunn, K. R., Am. Rev. Tuberc., 60, 439 (1949).
- Getz, H. R., Dunn, K. R., Dunn, M. S., Camien, M. N., Akawie, S., Malin, R. B., and Eiduson, S., Am. Rev. Tuberc., 60, 448 (1949).
- 7. Lankford, C. E., Swausch, C., and Ravel, J. M., J. Bact., 53, 368 (1947).
- 8. Hill, E. G., and Patton, A. R., Science, 105, 481 (1947).
- 9. Patton, A. R., and Hill, E. G., Science, 107, 68 (1948).
- Riesen, W. H., Spengler, H. H., Robblee, A. R., Hankes, L. V., and Elvehjem,
 C. A., J. Biol. Chem., 171, 731 (1947).
- Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., Arch. Biochem., 13, 207 (1947).
- Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., Univ. Calif. Pub. Physiol., 8, 293 (1949).
- 13. Camien, M. N., and Dunn, M. S., J. Biol. Chem., 173, 137 (1948).
- Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., J. Biol. Chem., 156, 703 (1944).
- Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., J. Biol. Chem., 168, 1 (1947).

⁷ That the responses in Fig. 2 are as good as they are after 20 and 40 minutes autoclaving may probably be attributed to the relatively low initial pH (6.32) of the medium. The browning of the medium is much more extensive at higher pH values, and the lability of the cystine would be expected to increase with the pH.

- 16. Speck, M. L., and Pitt, D. A., Science, 106, 420 (1947).
- 17. Camien, M. N., Dunn, M. S., and Salle, A. J., J. Biol. Chem., 168, 33 (1947).
- Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., J. Nutr.,
 33, 209 (1947).
- Woodson, H. W., Hier, S. W., Solomon, J. D., and Bergeim, O., J. Biol. Chem., 172, 613 (1948).

ANALYSIS FOR VITAMIN B₁₂ AND VITAMIN B_{12a} BY PAPER STRIP CHROMATOGRAPHY

By H. BOYD WOODRUFF AND JOHN C. FOSTER

(From the Research Laboratories, Merck and Company, Inc., Rahway, New Jersey)

(Received for publication, November 3, 1949)

Methods for separation of various growth factors, active for Lactobacillus lactis or Lactobacillus leichmannii, have been described. Chromatographic techniques employed by Lester Smith allowed separation of two red bands containing substances clinically active against pernicious anemia and having microbiological activity with L. lactis (1). Studies by Cuthbertson and Lester Smith with partition chromatography on paper, combined with microbiological assays on a solid medium, permitted demonstration of these two factors in concentrations of 0.005 to 0.1 γ . Water-saturated n-butanol was used as solvent with either upward or downward development (2).

A similar method, employed by Winsten and Eigen, showed double growth zones in the microbiological assay following partition chromatography of a concentrate of vitamin B_{12} and of a crystalline antipernicious anemia factor. Incomplete separation of the zones was obtained, the R_r values ranging between 0.0 to 0.03 and 0.03 to 0.10 for the two substances (3).

Additional substances which had growth-promoting activity for the assay organisms, but moved rapidly, were noted upon chromatographing liver extracts and animal protein factor concentrates. One of these substances had an R_F value characteristic of thymidine. Additional desoxyribosides, which have growth factor activity for L. leichmannii (4), may account for other zones found following the plating of a developed strip on a microbiological assay medium.

With the basic methods described above, we have been successful in defining conditions which allow quantitative separation of vitamins B_{12} and B_{12a} from mixtures of these two substances. The method has been applied with fermentation broths and liver concentrates for the separation of vitamin B_{12} - and B_{12a} -like materials from other microbiologically active growth factors.

Procedures

In our work, both ascending and descending methods of paper strip chromatography have been employed. The latter procedure is preferable, since extended diffusion times may be used to obtain complete separation. Whatman No. 1 filter paper was cut in strips $1 \times 22\frac{1}{2}$ inches. Experiments have been made with untreated strips or with buffered strips, as indicated below. Strips were impregnated with various salts by passage through 0.66 M solutions, followed by drying in air. The vessels used for development were large cylinders $6\frac{1}{2} \times 30$ inches, closed at one end, or a rectangular vessel $9\frac{1}{2} \times 29\frac{1}{2} \times 28\frac{1}{2}$ inches. Absorbent cotton moistened with water was placed in the bottom of each vessel. A dish containing water-saturated organic solvents was placed beneath the strips and a trough filled with solvent was used for immersion of the upper ends of the strips. The vessels were covered with a glass plate and sealed with silicone. Water-saturated solvents were employed in all cases. Benzyl alcohol, cresol, n-butanol, and secondary butanol were used in the survey experiments. A temperature of about 28°, in an area with little air movement, was maintained except where noted.

Strips were spotted with 0.01 ml. of a water or a solvent solution of material containing vitamin B_{12} . When the concentration of vitamin B_{12} was inadequate, successive spottings were made, and a continuous flow of warm air used to promote rapid drying. The minimum concentration of vitamin B_{12} or B_{12a} detectable was about 0.002 γ . Since diffusion rates were somewhat variable from day to day, control samples of pure vitamin B_{12} were always included.

Microbiological fermentation broths were extracted by techniques described in the literature to yield concentrated samples, for ease in application to the paper strip. Either butanol extraction from an $(NH_4)_2SO_4$ -saturated broth (5) or extraction with o-cresol (6) was successfully employed.

For the former extraction, 0.5 volume of water-saturated butanol was mixed with 1 volume of a butanol-saturated water solution of LLD factors. Excess $(NH_4)_2SO_4$ was added, followed by thorough mixing. The extraction was repeated until microbiological assay showed no further decrease in the LLD factor content of the broth. A measured portion of the butanol layer was spotted for development. In the cresol extraction, repeated treatment with 0.1 volume of o-cresol served to extract the major portion of vitamins B_{12} and B_{12a} .

After spotting, strips were placed in the saturated atmosphere of the solvent vessel for equilibration for 2 hours before solvent was added to the trough to initiate diffusion down the strip. Following development of the chromatogram with the solvent, strips were laid on large rectangular assay plates containing the inoculated medium employed in the cup assay

¹ Fermentation broths contain a variety of substances which produce a vitamin B_{12} -like response in microbiological assays with $L.\ lactis$ Dorner. These substances are called LLD factors (9).

with L. lactis Dorner (7, 8). After incubation overnight, strips were removed from the agar, revealing oval zones of growth beneath the location of LLD-active substances. Duplicate strips were sectioned and leached into 10 ml. of water to yield solutions for quantitative assay by the L. lactis Dorner titrimetric procedure (9).

EXPERIMENTAL

Plain paper strips were employed for preliminary experimentation with mixtures of vitamins B₁₂ and B_{12a}. No movement from the point of application was produced by development with benzyl alcohol. Movement but no separation was obtained with secondary butanol. The toxic effect of cresol prevented demonstration of microbiologically active areas on paper strips, even after attempts to remove the cresol with ether. n-Butanol development showed a double zone, similar to that described by Winsten and Eigen (3). Thymidine could readily be distinguished with

 $T_{ABLE} \ I \\ Chromatographic \ Development \ of \ Vitamins \ B_{12} \ and \ B_{12a} \ on \ Impregnated \ Strips$

	Impregnating salt								
Vitamin tested	Citric acid-Na citrate, pH 1.9	Citric acid-Na citrate, pH 3.0	KH ₂ PO ₄ , pH 4.6	NaCl					
		Diffusion, mm	from origin						
B _{12a}	0	7	18	70					
B ₁₂	0	23	58	71					

the latter solvent, since it moved rapidly along the strip with an R_F value of about 0.5.

Attempts were made to promote separation of vitamins B₁₂ and B_{12a} with n-butanol as a developing agent by impregnating strips with various salts at different pH levels (Table I). The time of development of strips with solvent was lengthened to 2 days to promote the separation. The best separation was obtained with strips impregnated with KH₂PO₄. No movement was obtained with plain paper strips or at pH 1.9. Although some separation was obtained at pH 3.0, the zones appeared as doublets. NaCl promoted diffusion but no separation.

More rapid separation was obtained with increased temperature. Strips impregnated with KH_2PO_4 showed LLD-active areas by microbiological assay for vitamin B_{12a} 45 mm. from the origin and for vitamin B_{12} 170 mm. from the origin following 45 hours development at 37°. This result may be compared with results recorded in Table I for development at 24° for a similar time period, or with a result of 57 mm. and 230 mm. at 24° for 72 hours.

Pure vitamin B_{12a} , obtained by hydrogenation of vitamin B_{12} (10), invariably produced a single well defined zone of growth under paper strips in the microbiological assay, as shown in Strip b, Fig. 1. Vitamin

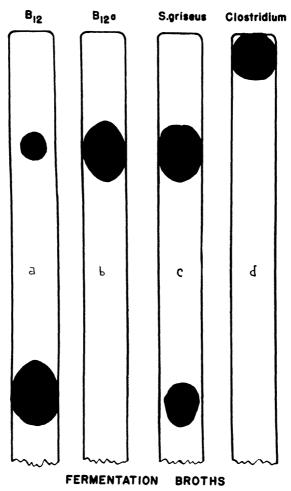


Fig. 1. Representative drawings of paper strip chromatographs. The inked spots are areas which promoted growth of L. lactis. Strips a, b, c, d are described in the text.

 B_{12} likewise showed a single growth zone when concentrations lower than 0.01 γ were placed on the strips. At higher concentrations of B_{12} , however, a second zone, having light growth, appeared also in the vitamin B_{12a} position (Fig. 1, Strip a). With mixtures of vitamin B_{12a} and high

concentrations of vitamin B_{12} , the vitamin B_{12a} zone and the second zone derived from vitamin B_{12} were superimposed, indicating that the second zone probably was vitamin B_{12a} . Additional evidence was obtained by comparative microbiological assay of the leachings from a strip spotted with a very high concentration of vitamin B_{12} (Table II). As will be noted, a low microbiological assay value with L. leichmannii, characteristic of vitamin B_{12a} (11), was found for the first zone obtained from vitamin B_{12} .

Crystalline vitamin B_{12} of 95 per cent purity, as determined by solubility analysis,² was used in these studies. It contained no chemically detectable amount of vitamin B_{12a} . Evidence that conversion of vitamin B_{12} to B_{12a} took place during the chromatographic analysis, and was not present in the original sample, was obtained by rechromatographing the leachings from the above experiment. The slow moving zone rechromat-

Table II

Comparative Microbiological Assay of Two Zones Derived from Vitamin B₁₂ by Paper

Strip Chromatography

	Distance from	Vitamin	B ₁₂ activity
Sample	origin	L. lactis assay	L. leichmannii assay
	mm.	γ	γ
Zone 1	43	1.1	0.41
" 2 Crystalline vitamin B_{12a} , per	196	15.2	13.0
gamma		0.9	0.2
Crystalline vitamin B ₁₂ , per gamma		1.0	1.0

ographed into a single zone, which is characteristic of vitamin B_{12a} . The rapid moving zone rechromatographed into two zones, the major one being vitamin B_{12} and the minor one characteristic of vitamin B_{12a} . Leaching of the rechromatographed zones, followed by a third chromatographing, gave similar results.

Over the normal range of working conditions of the method, a fairly constant proportion of vitamin B_{12a} is obtained from vitamin B_{12} . It has been our practice to assume a correction factor based on conversion of 10 per cent of the observed vitamin B_{12} to B_{12a} during the chromatographic procedure, since approximately this degree of conversion was obtained in control experiments. Table III presents observed vitamin B_{12a} percentages obtained from chromatography of various concentrations of vitamin

 $^{^2}$ We wish to thank Mr. F. A. Bacher for solubility analysis and solvent distribution studies on the vitamin B_{12} preparation.

 B_{12} and presents recovery data which demonstrate the quantitative nature of the procedure.

The procedure has been used for estimation of the concentration of vitamins B_{12} and B_{12a} -like materials produced by microorganisms. Adequate concentrations of activity for spotting were obtained by solvent extraction procedures. The assay has also been applied to concentrates

Table III

Quantitative Microbiological Assay of B_{12a} Zone from Chromatography of Various

Quantities of Vitamin B_{12}

Vitamin Bu equ	Per cent of vitamin B ₁₂ chromatographed found in	
Vitamin B _{12a} zone	Vitamin B ₁₂ zone	vitamin B _{12a} zone
γ	γ	
7.9	84	8.7
7.0	55	10.3
5.0	37	11.1
2.6	21	11.8
	Vitamin B _{13a} zone 7 7.9 7.0 5.0	7 7.9 84 7.0 55 5.0 37

Table IV

Analysis of Fermentation Broths and Antipernicious Anemia Concentrate by Paper
Strip Chromatography

						ctionatic ological a	
		Culture		Nitrogen source	Solvent soluble	Vitamin B ₁₂ zone	Vitamin B ₁₂ zone
\bar{S} .	griseus	strai	n 1	Whole protein medium	51	18	1.9
"	"	"	1	Protein hydrolysate medium	69	34	<0.4
"	"	"	1	Synthetic medium	70	<2	15
"	"	"	2	Protein hydrolysate medium	80	24	1.5
Stre	eptomy	ices sp			67	20	< 0.4
	tetano	•		Brain-heart medium	53	<2	<2
		•		15 U.S. P. unit liver extract	†	97	<3.0

^{*} The figures represent the per cent of L. lactis Dorner activity of the original sample recovered in various fractions.

obtained from fermentation sources, which have antipernicious anemia activity, and to U. S. P. liver concentrates. These substances contain sufficient activity so that they may be applied directly to the paper strip without preliminary solvent extraction. Examples of the results of assays are presented in Table IV.

There was great variation in the proportion of total vitamin B₁₂ to

[†] Sample spotted directly on strips without solvent extraction.

 B_{12a} type of compounds found in different fermentation broths by paper strip chromatography in relation to the microbiological LLD activity of the broths. Compounds having R_F values similar to vitamin B_{12} or to vitamin B_{12a} are present in some fermentation broths, but these compounds may represent nearly all or less than 10 per cent of the microbiologically active growth factors for L. lactis contained in the broth. The culture medium and the microorganism both are influential in determining the concentration and proportion of the growth factors isolated. Table IV shows evidence of the range of results obtained from the growth of a single strain of Streptomyces griseus on three media. Also, variation is noted among several cultures. Fig. 1, Strip c, represents the indicator strip obtained with fermentation broth of S. griseus (strain 2) grown on a protein hydrolysate medium. LLD titrimetric assay showed 1.5 per cent of the original broth activity to be in the vitamin B_{12} zone and 24 per cent in the vitamin B_{12a} zone.

Thymidine and related compounds which have LLD activity are present in fermentation broths, but they are leached off the paper by chromatographic technique. An additional microbiologically active compound which is retained on the paper strip is present in some fermentation broths. Fig. 1, Strip d, represents the chromatographic analysis of solvent-extractable material produced by a species of Clostridium. The quantitative data obtained with this culture are presented in Table IV. Less than 2 per cent of the LLD activity of the broth was found to have R_F values similar to those for vitamin B₁₂ or B_{12a}, but approximately half the LLD activity was retained at the point of application on the paper strip. Solvent concentrates from this culture, containing the non-moving microbiologically active factor, yielded negative results when tested for vitamin B₁₂-like growth-promoting activity in chicks. With some cultures the non-moving zone and the vitamin B_{12a} zone form a doublet, making it impossible to obtain a quantitative evaluation for the vitamin B_{12a} type of material.

A liver extract for parenteral use in pernicious anemia (15 U. S. P. units per ml.) was found to contain activity equivalent to 17 γ of vitamin B₁₂ per ml. by titrimetric assay with L. lactis. However, on paper strip analysis no vitamin B₁₂ was present, but activity equivalent to 16.5 γ of vitamin B_{12a} was contained in the vitamin B_{12a} area of the paper strip. The indicator strip for this sample was indistinguishable from that represented in Fig. 1, Strip b, for vitamin B_{12a}.

SUMMARY

A method has been presented for quantitative estimation of vitamins B_{12} and B_{12a} , combining microbiological assay and paper strip chromatog-

raphy. A small corrective factor is required to compensate for conversion of vitamin B_{12} to vitamin B_{12a} , which may take place during the analysis. The method has been applied to microbiological fermentation broths and to injectable liver extracts (U. S. P.) for the analysis for vitamin B_{12} and B_{12a} type of materials. No quantitative relationship was found between the amount of these vitamins contained in the samples and the *Lactobacillus lactis* titrimetric assay of the samples.

BIBLIOGRAPHY

- 1. Lester Smith, E., Nature, 161, 638 (1948).
- 2. Cuthbertson, W. F. J., and Lester Smith, E., Biochem. J., 44, p. v (1949).
- 3. Winsten, W. A., and Eigen, E., J. Biol. Chem., 177, 989 (1949).
- 4. Kitay, E., McNutt, W. S., and Snell, E. E., J. Biol. Chem., 177, 993 (1949).
- 5. Ellis, B., Petrow, V., and Snook, G. F., J. Pharm. and Pharmacol., 1, 61 (1949).
- Wolf, D. E., Wood, T. R., Valiant, J., and Folkers, K., Proc. Soc. Exp. Biol. and Med., in press.
- 7. Cuthbertson, W. F. J., Biochem. J., 44, p. v (1949).
- 8. Foster, J. C., Lally, J., and Woodruff, H. B., Science, 110, 507 (1949).
- Caswell, M. C., Koditschek, L. K., and Hendlin, D., J. Biol. Chem., 180, 125 (1949).
- 10. Kaczka, E., Wolf, D. E., and Folkers, K., J. Am. Chem. Soc., 71, 1514 (1949).
- Henlin, D., and Woodruff, H. B., Abstracts, American Chemical Society, 116th meeting, Atlantic City, 33A (1949).

ALTERATIONS IN BLOOD GLUCOSE FOLLOWING INTRA-VENOUS GALACTOSE

By LAWRENCE GREENMAN

(From the Department of Research Medicine, Children's and Presbyterian Hospitals, and Renziehausen Foundation, University of Pittsburgh School of Medicine, Pittsburgh)

(Received for publication, July 27, 1949)

No uniformity of response is evident in the reports of blood glucose concentrations observed following the administration of galactose. Oral administration of the latter sugar has been reported to elevate (1-4), depress (5-7), or not alter the blood glucose levels of healthy adults (8, 9), to increase that of subjects with diabetes mellitus (5, 10, 11), and to decrease greatly that of infants with idiopathic intolerance to galactose (12-17). The few studies with healthy infants indicate little or no change in glucosemia (12, 15). Finally no data have been found on the response of the blood glucose to galactose following its *intravenous* injection in infants or in adults with decreased utilization of the latter sugar.

To clarify the relationship between galactose and glucose the former sugar has been administered intravenously, in amounts usually employed in galactose tolerance tests, to thirteen healthy adults, five convalescent infants, four subjects with diabetes mellitus, thirteen adults with impaired utilization of galactose, and to one infant with idiopathic galactose intolerance. Since a major object of the study was to determine accurately the rate of utilization of galactose as well as alterations of blood glucose, the methods of analysis and calculation were carefully defined in control studies. As a result, a possible explanation for the differences in previous reports of changes in blood glucose has appeared.

Subjects and Methods

The subjects ranged in age from 8 weeks to 57 years. Activity was limited during the test. Galactose was injected intravenously as a 50 per cent solution in water in 3 to 7 minutes during the postabsorptive state. The infants received 1.2 gm. of the sugar per kilo of body weight; the others were given 0.4 to 0.6 gm. per kilo of body weight. Insulin was withheld the morning of the test from the patients with diabetes mellitus. None of them had acetonuria. The procedure was modified in seven of the healthy adults by the oral administration of 0.3 ml. of 95 per cent ethyl alcohol per kilo of body weight, 15 minutes before injection of the

sugar (18). This resulted in impairment of their ability to utilize galactose. In addition one infant with idiopathic intolerance to this sugar received alcohol in two studies in doses of 0.4 and 0.5 ml. per kilo of body weight 15 minutes before injection of galactose, and on one other occasion crystalline insulin, 2 units subcutaneously. Further information concerning these patients and their galactose metabolism has been previously presented (18, 19).

Venous blood was collected in bottles containing oxalate immediately before the injection and then at 15, 45, and 75 minutes after its mid-point. Two analytical procedures were employed; Somogyi's iodometric titration method for galactose because of its accuracy in the low concentrations encountered (20), and Benedict's method for total sugar because of its speed, simplicity, and reliability in the ranges analyzed (21). Galactose alone was not measured by Benedict's method, since the procedure is not dependable at concentrations below 40 mg. per cent, and consequently the 15 minute sample of blood would have been the only one in which the values of this sugar would have been consistently in the range for accurate measurement.

The samples for galactose determination were fermented with 6.0 ml. of 20 per cent bakers' yeast for \frac{1}{2} hour at room temperature. Control studies established the adequacy of these conditions to remove glucose without altering galactose. Prior to its use the yeast was washed six times with distilled water, and the hematocrit of each yeast suspension determined. Yeast blanks were found to be identical with water blanks when analyzed by the Somogyi procedure. Following the precipitation of protein from the glucose-free blood by zinc sulfate and sodium hydroxide (22), the filtrate was analyzed in duplicate for galactose. A reaction time of 25 minutes with the alkaline copper reagent was employed. to 100 per cent of this sugar was recovered from known solutions. blood specimen obtained immediately before the administration of galactose served as a blank for non-glucose-reducing substances remaining after fermentation, and was taken into account in all calculations of galactose values (18). All dilution factors, including the addition of the yeast (corrected for its hematocrit), were considered in the final calculation of the galactose levels.

The second 2.0 ml. aliquot of blood was not fermented, but was analyzed in duplicate for total sugar (glucose plus galactose) with a Somogyi filtrate (22). In most of these determinations the sugars were permitted to react with the Benedict alkaline copper reagent for 5 minutes. Glucose plus galactose was thus measured together, but galactose had only 65 per cent of the reducing power of glucose under these conditions. Further analysis of the relationship between reaction time and recovery of

galactose revealed that the reducing power of galactose increased to 80 per cent of that of glucose, when the time ranged from 10 to 15 minutes. Glucose reduction was complete during all of these intervals. The findings are presented in Table I. They are consistent with the principles concerning copper reduction methods for sugar analyses described by Somogyi (20). It is clear that the time of reaction with the alkaline copper reagent must be carefully controlled if reproducible recoveries of galactose are to be obtained with a 5 minute period. The twenty-six experiments listed in the first part of Table I illustrate the accuracy with which this interval may be employed.

TABLE I

Recovery of Galactose by Benedict Sugar Method; 100 Mg. Per Cent Glucose As Standard and Varying Time of Reaction with Alkaline Copper Reagent

Total sugar,	Total sugar, galactose + glucose		Total sugar	Galactose*	Galactose	
Reaction time	mg. per cent	experiments	recovered	Garactose	recovered	
win.			mg per cent	mg per cent	per cent	
5	50 + 100	2	133	33	66	
	75 + 100	2	148	48	64	
i	100 + 100	3	162	62	62	
	150 + 100	3	198	98	65	
ŀ	200 + 100	3	230	130	65	
i	100 + 0	4	68	68	68	
	150 + 0	5	98	98	65	
1	200 + 0	4	131	131	65	
10	100 + 0	3	80	80	80	
15	100 + 0	3	80	80	80	

^{*} Galactose calculated as total sugar minus glucose.

Calculation of Blood Glucose—It is evident that blood glucose concentrations were not measured directly but had to be calculated from the total sugar value and the galactose concentration in the following manner.

Glucose = total reducing capacity - 65 % galactose concentration

For example:

Total reducing capacity (Benedict method, 5 min. reaction time) = 204 mg. % Galactose (Somogyi method) = 147 mg. % Glucose = $204 - (0.65 \times 147) = 108$ mg. %

If Benedict's method with glucose as the standard were employed for measurement of both "galactose alone" and "total sugar" with a reaction time of 10 minutes, the following calculations would be necessary:

```
Total sugar (as glucose) = 140 mg. % Residual reducing power (as glucose, following fermentation by yeast) = 80 mg. % Actual galactose concentration = 80/0.8 = 100 mg. % Glucose = 140 - 80 = 60 mg. % but not 140 - 100 = 40 mg. %
```

In certain ranges this erroneous calculation will either mask elevations of blood glucose or indicate large declines in this sugar when none has occurred. The general application of this concept to other sugar methods such as those of Somogyi, Hagedorn-Jensen, and Folin-Wu, in which galactose and glucose differ from each other in reducing power, is clear (20, 23, 24).

Results

Carbohydrate concentrations of the blood of the subjects with normal galactose tolerance tests are presented in Fig. 1 and Table II. The healthy adults, with one possible exception (L. G.), had significant elevations of blood glucose, *i.e.* 10 per cent or greater, which were usually evident at 15 minutes. In one instance (J. R.) the maximum elevation was not observed for 45 minutes, and in one other (J. P.) until 75 minutes. Similar rises occurred in the diabetics and the convalescent infants. The increase was greatest in the infants, presumably as the result of the large dose of galactose. Following this elevation, glucose concentrations frequently fell to levels below the initial ones, except in the diabetic patients.

The infant with the greatest inability to utilize galactose had no increase in blood glucose on three occasions, although blood levels did not fall below 50 mg. per cent following galactose alone (Fig. 1, Table III). When he received alcohol or insulin beforehand, however, hypoglucosemia with its attendant symptoms appeared despite an elevated total sugar level. In contrast to J. S., the subjects with galactose tolerances impaired by the administration of alcohol did not develop hypoglucosemia. None of their glucose values differed significantly from those of the healthy adults without alcohol; although the response was more variable (Fig. 1, Table III).

The pattern of alteration of blood glucose in the subjects with hepatic disease differed from that of the healthy adults, since elevations were not evident at the 15 minute point, but were delayed (Fig. 1, Table III).

DISCUSSION

Blood glucose alterations following the rapid intravenous injection of galactose are related to the subject's ability to utilize, convert, store, or excrete the latter sugar. The relationship is complex, however, since the disappearance of galactose is a function of several processes, the rates

of each of which presumably vary with time; furthermore, changes in blood glucose must be the result of the rates of formation and utilization of glucose. Elevations of blood glucose concentrations, therefore, have appeared when the rate of entry of glucose into the blood stream and body fluids exceeded its rate of removal from these compartments. Galac-

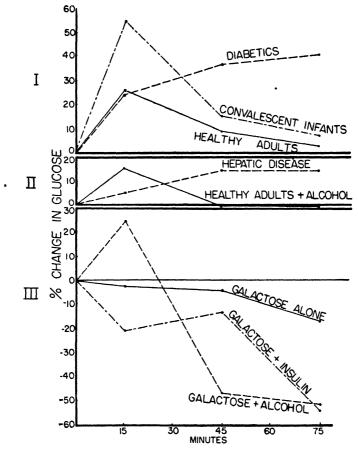


Fig. 1. Mean per cent changes in blood glucose. Section I, patients with normal galactose tolerance tests; Section II, adults with impaired ability to utilize galactose; and Section III, infant with idiopathic galactose intolerance.

tose has entered this sequence through its conversion into glycogen and glucose in the liver (25-29). Confirmatory evidence of the rôle of glucose utilization in this mechanism has appeared in reports that serum inorganic phosphorous levels fall (30), the R. Q. rises (31, 32), and lactic acid production increases (33) following administration of galactose to

Table II

Total Sugar and Glucose Response of Subjects; Normal Intravenous Galactose Tolerance Tests

				u	nce.	1 6818								
Patient	Sex	Age	Weight	Galac- tose ad- minis- tered	0	Total	sugar 45	75	G 15	alacto	se 75	15	Glucos	e 75
	1	ļ	¥.	per kilo	min.		min	min.					min.	min.
				He	althy	adu	lts						_	
		yrs	kg.	gm.	mg per cent	mg. per cent								
B. M.*	M.	33	91	0.6	86	274	155	115	218	103	27	132	88	97
J. R.	1 "	33	70	0.6	80	179	120	94	150	30	10	81	100	86
L. G.†	1 "	27	68	0.6	88	171	100	84	125	27	12	90	82	76
J. P.	1 "	31	66	0.6	80	200	140	104	171	76	10	89	91	97
J. M.	\mathbf{F}	19	57	0.6	75	230	117	86	209	72	12	94	70	78
B. K. (a)	1 "	22	60	0.6	87	238	139		i	74		114	91	89
" $"$ (b)	"	22	60	0.4	85		99	90		18	6	94	87	86
F. M.‡	M.	27	63	0.5	76	234	153	96	152	50	16	112	113	83
J. T. (a)	"	31	77	0.5	79	204	128	96	147		16	108	92	86
" $"$ (b)	"	31	77	0.5	84	209	118	94	155	53	15	108	84	84
H. W.	F.	12	44	0.5	85	177	108	87	117	24	7	101	92	82
D. G.	"	23	52	0.5	86	188	95	79	131	7	3	103	90	77
I. L.	"	24	54	0.5	73	193		88	150		19	96		76
L. F.	"	35	50	0.5	72	182		84	120		12	104		76
M. C.	"	27	50	0.5	82	209	123	88	167	57	11	100	86	81
				Diab	etes	mell	itus			-				
M. S.	F.	15	43	0.5	66	158	120	119	95	12	3	96	112	117
M. B.	"	14	42	0.5	222	339	281	287	125	35	18	258	258	275
T. V.‡	M.	13	44	0.5	264	349	305	306	83	18	0	283	291	306
E. O.‡	"	14	53	0.5	141	276	229	209	108	25	5	190	209	205
		·	1.37	Conva	lesce	nt in	fant	3						
	1	uks.												
D. C.	F.	8	5.0	1.2	80	254	144	94	157	23	13	152	129	86
R. D.	M.	8	5.3	1.2	66	228	110	82	204	50	12	95	78	74
J. F.	"	16	3.7	1.2	73	230	92	88	212	48	8	92	61	83
C. Q.	"	18	4.4	1.2	62	246	108	72	205	64	15	113	66	62
G. H.	F.	28	5.4	1.2	76	216	134	88	174	78	15	103	83	78
* Pagaired	54 6	am .	of gold	natoso				· · · · · ·	·····					

^{*} Received 54.6 gm. of galactose.

subjects with normal ability to utilize glucose, while these changes are not observed in diabetic subjects (34, 35).¹ In addition the temporary

^{† 30} minutes total sugar = 133 mg. per cent; glucose = 95.

[‡] Factor for conversion of galactose into its glucose equivalent = 0.8 in these experiments.

¹ Greenman, L., unpublished data.

Table III

Total Sugar and Glucose Response of Subjects; Impaired Intravenous Galactose

Tolerance Tests

.			. .	Galac- tose ad-		Total	sugar	•	C	alacto	se		Glucos	se
Patient	Sex	Age	Weight	minis- tered per kilo	0 min.	15 min.	45 min.	75 min.	15 min.	45 min.	75 min.	15 min.	45 min.	7.5 mi
			Hea	lthy adu	ılts f	ollov	ving	alcol	nol					
		yrs.	kg.	gm.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg pe cen
J. R.	M.	33	70	0.6	97	242	173	145	195	111	59	115	101	107
L. G.	"	27	68	0.6	82	181	133	117	152	94	65	82	72	78
J. M.	F.	19	57	0.6	80	219	152	123	184	134	85	99	65	6
B. K.	"	22	60	0.5	81	202	154	122	182	121	82	84	75	69
J. T.	M.	31	77	0.5	81	192	156	137	179	106	74	76	87	89
I. L.	F.	24	54	0.5	81	180	}	124	156		68	79		8
M. C.	"	27	50	0.5	93	234		107	163		54	128		7:
			Hep	atic dis	ease;	gala	ctos	e alo	ne		Accessed to wood			
J. C.*	M.	50	66	0.5	73	164	140	112	144	84	42	70	85	8
L. B.*	"	48	78	0.5	124	240		200	159		88	137		143
M. T.*	F.	57	48	0.5	87	172		103	132	ļ	23	86		8
W. P.†	M.	54	76	0.5	72	224	149	112	206	88	52	90	92	7
D. H.;	"	53	67	0.5	80	209	161	139	151	85	25	88	93	119
E. K.§	$\mathbf{F}.\ $	50	48	0.5	75	160	121	98	111	53	23	71	79	80
		Infa	nt wit	h idiop	athic	gals	ctos	e int	olera	nce				
		wks.										1		
J. S. (galac-	M.	21	5.0	1.2	72	207	187	151	222	170	155	63	76	50
tose alone)	Ì	24	5.3	1.2	70	224	178	166	233	180	157	73	61	64
		27	5.5	1.2	77	255	207	1	272	208	188	78	72	68
J.S. (galac-	M.	29	6.0	1.2	67	234	146	130	218	188	168	92	24	2
tose + al- cohol)		41	8.1	1.2	70	25 0	178	154	234	202	169	98	47	4
,	М.	26	5.6	1.2	48	164	132	100	194	138	122	38	42	2

^{*} Cirrhosis of liver.

increase in blood glucose levels following galactose administration may be prevented by accelerating glucose utilization with insulin.¹

Similar changes of blood glucose in the healthy adults in the experi-

[†] Amyloidosis.

[‡] Convalescent from homologous serum jaundice.

[§] Biliary cirrhosis.

^{||} Factor for conversion of galactose into its glucose equivalent 0.8 in these experiments.

ments with, and those without, alcohol, despite significantly different galactose tolerance tests, cannot be definitely explained, although they seem to have been the result of the time interval required for alcohol to impair galactose utilization. In addition, no satisfactory explanation is apparent for the severe hypoglucosemia observed in the infant with idiopathic galactosuria when alcohol was administered with the galactose, especially since neither alcohol alone nor glucose alone evoked such a response.

Despite the variety of changes in glucosemia which may appear following the administration of galactose alone, it is not likely that profound decreases in blood glucose levels will occur. Reports indicating this occurrence must be reexamined (5-7, 12, 14-17), especially since the absence of symptoms of hypoglucosemia in infants with idiopathic intolerance to galactose has been ascribed to simultaneously elevated total blood sugars (glucose plus galactose). This observation has provided strong support for the theory that symptoms of hypoglucosemia do not necessarily depend on low blood glucose values, but rather on the level of total blood sugar. The explanation for these findings apparently lies in the failure to account for the different reducing powers of both sugars (12, 14-17). Recalculation of the data in these infants may explain their anomalous condition, since blood glucose levels then appear higher. interpretation is confirmed by observations that galactose will not alleviate a severe insulin reaction despite the presence of an increased total blood sugar (5, 9), and that the infant with galactosuria presented here as well as the one described by Norman and Fashena (17) demonstrated symptoms of hypoglucosemia even though the total sugar was elevated.

SUMMARY

Alterations of blood glucose following the rapid intravenous injection of galactose are related to the subject's ability to metabolize the latter sugar, and depend on the resultant of the rate of entry of glucose into the blood stream and body fluids and its rate of removal from these compartments. The different reducing powers of glucose and galactose must enter into any calculation of the amounts of these two sugars, when they are present simultaneously in the blood stream. Although blood glucose values may rise, remain unchanged, or fall slightly following galactose administration, it seems unlikely that galactose alone will produce severe hypoglucosemia.

The author expresses his appreciation to Dr. T. S. Danowski for his valuable aid and advice.

BIBLIOGRAPHY

- 1. Maclagan, N. F., Quart. J. Med., 9, 151 (1940).
- 2. Harding, V. J., and van Nostrand, F. H., J. Biol. Chem., 85, 765 (1929-30).
- 3. Bakx, C. J. A., Nederl. Tijdschr. Geneesk., 90, 961 (1946).
- 4. Harding, V. J., and Grant, G. A., J. Biol. Chem., 99, 629 (1932-33).
- 5. Roe, J. H., and Schwartzman, A. S., J. Biol. Chem., 96, 717 (1932).
- 6. Pierce, H. B., J. Nutr., 10, 689 (1935).
- 7. Wagner, R., Am. J. Dis. Child., 65, 207 (1943).
- 8. Cori, C. F., and Cori, G. T., Proc. Soc. Exp. Biol. and Med., 25, 402 (1928).
- 9. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Interpretations, Baltimore, 2nd edition, 1 (1946).
- 10. Koehler, A. E., Rapp, I., and Hill, E., J. Nutr., 9, 715 (1935).
- 11. Bollman, J. L., and Mann, F. C., Am. J. Physiol., 107, 183 (1934).
- 12. Mason, II. H., and Turner, M. E., Am. J. Dis. Child., 50, 359 (1935).
- 13. Talbot, N. B., Crawford, J. D., and Bailey, C. C., Pediatrics, 1, 337 (1948).
- 14. Goldstein, E. O., and Ennis, J. M., J. Pediat., 33, 147 (1948).
- 15. Goldbloom, A., and Brickman, H. F., J. Pediat., 28, 674 (1946).
- Bruck, E., and Rapoport, S., Am. J. Dis. Child., 70, 267 (1945).
 Norman, F. A., and Fashena, G. J., Am. J. Dis. Child., 66, 531 (1943).
- Greenman, L., Tipping, J. S., and Rosenbaum, J. D., Am. J. Med. Sc., 217, 644 (1949).
- 19. Greenman, L., and Rathbun, J. C., Pediatrics, 1, 666 (1948).
- 20. Somogyi, M., J. Biol. Chem., 160, 61 (1945).
- 21. Benedict, S. R., J. Biol. Chem., 68, 759 (1926).
- 22. Somogyi, M., J. Biol. Chem., 86, 655 (1930).
- 23. Hagedorn, H. C., and Jensen, B. N., Biochem. Z., 135, 46 (1923).
- 24. Folin, O., J. Biol. Chem., 77, 421 (1928).
- 25. Deuel, H. J., Jr., Physiol. Rev., 16, 173 (1936).
- 26. Bell, D. J., Biochem. J., 30, 1612 (1936).
- 27. Kosterlitz, H. W., and Ritchie, C. M., Biochem. J., 37, 618 (1943).
- 28. Draudt, L., Arch. Exp. Path. u. Pharmakol., 72, 457 (1913).
- 29. Stetten, D., Jr., J. Biol. Chem., 159, 123 (1945).
- 30. Free, A. H., and Leonards, J. R., J. Biol. Chem., 149, 203 (1943).
- 31. Wierzuchowski, M., Biochem. Z., 230, 187 (1931).
- 32. Carpenter, T. M., and Lee, R. C., Am. J. Physiol., 102, 635 (1932).
- 33. Wierzuchowski, M., and Laniewski, M., Biochem. Z., 230, 173 (1931).
- McCullagh, E. P., and McCullagh, D. R., J. Lab. and Clin. Med., 17, 754 (1931–32).
- 35. Roe, J. H., Gilman, A., and Cowgill, G. R., Am. J. Physiol., 110, 531 (1935).

ON THE ACTIVITY OF OXYBIOTIN*

By RICHARD L. POTTER AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, November 16, 1949)

Recent advances in our knowledge of the metabolic activities of biotin (1-6) have increased interest in its biologically active analogue, oxybiotin. It is known that oxybiotin has biotin-like activity for several species of bacteria (7-10), yeast (7), and rats and chickens (11). In those cases which have been adequately tested (9, 12-14), it has been clearly demonstrated that oxybiotin is active per se and is not converted to biotin. In fact it has recently been suggested that oxybiotin might have biotin-like activity for all biological forms (15). However, since most of the early biological tests on oxybiotin were carried out before the multiple activities of biotin were known, it appears that the capacity of oxybiotin to replace biotin in every case cannot be inferred from its ability to carry out some of the functions of biotin. Two of the better known activities of biotin are its capacity to replace the need for oleic acid (16-18) and aspartic acid (1, 18, 19) for certain lactic acid bacteria. When biotin replaces aspartic acid, it causes aspartic acid synthesis (19), and it has been suggested that the mechanism involves carbon dioxide fixation (2, 3, 18, 20).

It is well known that oxybiotin is as active or nearly as active as biotin for a number of microorganisms in the presence of aspartic acid (7-10, 21-23). However, Stokes et al. (19) have indicated that the ability of oxybiotin to replace biotin in the absence of aspartic acid is negligible in the case of Lactobacillus casei and is only 0.2 to 1.0 per cent as great as biotin in the case of Lactobacillus arabinosus 17-5. For both of these organisms, oxybiotin shows a very high activity in the presence of aspartic acid. This brief report is presented to show that small changes in the initial pH of the bacterial medium will cause the apparent activity of oxybiotin in the absence of aspartic acid to vary several hundred fold and that these changes will mean the difference between poor and excellent growth during the usual type of microbiological test.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board.

EXPERIMENTAL

Methods and Materials

The basal medium consisted of glucose, acetate, amino acids, salts, purines, pyrimidines, and water-soluble vitamins (18). In order to obtain growth, the medium must be supplemented with either a small amount of biotin (0.5 mµgm, per 10 ml. of culture) and aspartic acid or a relatively large amount of biotin (20 mµgm.). The culture medium consists of 5 ml. of the basal medium supplemented as described and diluted to 10 ml. with distilled water. The assays were carried out in large size Evelyn colorimeter tubes plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure. The inoculum was a dilute washed saline suspension of a 12 to 24 hour culture of L. arabinosus 17-5. The growth was measured as turbidity (per cent transmission) with a tube of sterile culture medium as the blank or by titrating the acid produced with 0.1 N sodium hydroxide. The pH of the bacterial medium, a primary consideration in these experiments, was measured with a Beckman pH meter in preference to the less accurate visual methods. The term, initial pH, refers to the pH of the medium after autoclaving. The pH of the medium will decrease 0.1 to 0.2 upon autoclaving, if the pH is about 6.5, and somewhat more if it is above 7.0.

Results

It has been shown previously that in the presence of aspartic acid oxybiotin is 100 per cent (7, 21, 23) or 58 to 88 per cent (22) as active as biotin for L. arabinosus. The results of a typical experiment are shown in Table I. While the activity of the two compounds did not agree as closely as this in every experiment, the actual differences were small and did not appear to be related to the initial pH of the medium.

In contrast to these results the growth response of L. arabinosus to biotin and oxybiotin at pH 6.5 in the absence of aspartic acid is shown in Table II. Here the biotin requirement is greatly increased (18, 19) and the initiation of growth is delayed (19). For the organisms receiving biotin maximum growth is attained in 38 hours. For the organisms supplemented with oxybiotin the onset of growth is greatly retarded and only mediocre growth is attained in 66 hours. The best growth is only about one-fifth of the maximum and oxybiotin does not appear to be the limiting factor since 2000 m μ gm. give no better growth than 20 m μ gm. These results are perhaps comparable to those of Stokes, Larsen, and Gunness (19) which indicated that 100 to 500 times as much oxybiotin

¹ All expressions of activity and amounts of oxybiotin in this paper are made on the basis of the d isomer of the dl compound.

is necessary in the absence of aspartic acid. Their medium was adjusted to pH 6.8 before autoclaving.

Table I
Comparison of Biotin and Oxybiotin in Presence of Aspartic Acid, Initial pH 6.6

	Bio	otin	Oxybiotin			
	Turbidity* at 24 hrs.	0.1 N acid at 66 hrs.	Turbidity at 24 hrs.	0.1 N acid at 66 hrs.		
mµgm. per 10 ml.		ml.		mi.		
0	85	4.0	85	4.0		
0.125	76	8.7	77	8.6		
0.25	71	10.2	. 72	10.0		
0.375	63	11.9	66	11.8		
0.5	61	13.0	61	12.9		
0.625	58	13.9	58	13.9		
0.75	55	14.6	55	14.6		
1.0	50	15.5	50	15.4		
1.25	46	17.1	48	17.0		

^{*} Per cent transmission in the Evelyn colorimeter. A reading of 100 indicates absence of growth.

Table II

Activity of Biotin and Oxybiotin in Absence of Aspartic Acid, Initial pH 6.5

Oxybiotin

	•	Jioun				OAYL						
	7	Furbidity	,•	0.1 N acid produced	Addition of the second	Turbidity*			0.1 N acid			
	24 hrs.	38 hrs.	66 hrs.	at 66 hrs.		24 hrs.	38 hrs.	66 hrs.	at 66 hrs.			
mugm. per 10 ml.				ml.	mugm. per 10 ml.				ml.			
0	99	98	98	1.0	0	99	99	98	0.7			
0.5	97	96	96	1.3	0.5	97	95	94	1.1			
1.0	95	93	93	1.6	1.0	99	96	93	1.2			
2.5	92	87	87	2.7	5.0	98	94	91	1.7			
5.0	87	74	74	4.6	20.0	98	96	83	2.4			
10.0	86	55	54	10.0	250.0	99	96	82	2.4			
15.0	84	44	42	13.3	2,000.0	98	93	82	2.9			
20.0	86	36	32	14.6	20,000.0	100	99	98	0.7			

^{*} See foot-note to Table I.

Riotin

Table III presents the results of an experiment in which the initial pH was 7.0. It is evident that a more alkaline medium greatly favors the early growth of organisms supplemented with biotin and that the prolonged lag period previously recorded (19) for organisms synthesizing their own aspartic acid is eliminated. For organisms receiving oxybio-

tin, the lag period is considerably reduced but not eliminated. While growth is negligible at 24 hours, it is good at 38 hours and excellent growth has been obtained with moderate amounts of oxybiotin at 66 hours. By comparing the turbidity of the organisms at 66 hours, it is apparent that the response to 10 m μ gm. of oxybiotin falls between that obtained with 2.5 and 5.0 m μ gm. of biotin, and that 25 m μ gm. of oxybiotin give growth intermediate between that obtained with 5 and 10 m μ gm. of biotin. In terms of optical density the activity of oxybiotin is only slightly less than 30 per cent of that of biotin in both cases. Similar results have been obtained with an initial pH of 8.0 in the presence of 0.01 m bicarbonate and a gas mixture of 95 per cent nitrogen and 5 per cent carbon dioxide in a vacuum desiccator. In this experiment the turbidity of the tubes

Table III

Relative Activity of Biotin and Oxybiotin in Absence of Aspartic Acid, Initial pH 7.0

	Biotin			Oxybiotin					
		Turbidity*				Turbidity*			
	24 hrs.	38 hrs.	66 hrs		24 hrs.	38 hrs.	66 hrs.		
mugm. per 10 ml.	-			тµgm. per 10 ml.					
0.0	100	99	99	0.0	100	99	99		
2.5	86	74	64	2.5	98	94	93		
5.0	63	47	42	10.0	96	57	53		
7.5	47	36	32	25.0	97	5 8	36		
10.0	39	29	25	250.0	97	52	28		
15.0	30	24	20	1,000.0	98	54	27		
20.0	26	22	18	5,000.0	97	56	26		
25.0	33	22	18	25,000.0	97	97	24		

^{*} See foot-note to Table I.

containing oxybiotin was as great as 66 per cent transmission at 24 hours, although the activity compared to biotin at 66 hours was not greater than 25 per cent.

DISCUSSION

The results presented here indicate that oxybiotin is less effective than biotin in causing aspartic acid synthesis. The authors have shown previously (18) that, even in the presence of bicarbonate and biotin, aspartic acid cannot be synthesized at pH 5.8 or below. The data in Tables II and III indicate that the quantitative biotin requirement is largely a function of the pH. Why slightly acid conditions affect oxybiotin activity so much more adversely than that of biotin is not known, although it is

presumably related to the effect on bicarbonate concentration. Oxybiotin seems to be inherently less effective than biotin for carbon dioxide fixation even at neutral pH. Under acid conditions, which increase the lag period with biotin, the difference in activity of these two compounds is exaggerated to such an extent that good growth is not attained with oxybiotin during the normal assay period.

Wyss, Lilly, and Leonian (24) have shown that pH has a pronounced effect on the requirement of a *Neurospora crassa* mutant for *p*-aminobenzoic acid. From the dissociation constant of the vitamin and their experimental data, the authors conclude that only the molecular form can be utilized by this organism. This does not appear to explain the effect of pH on oxybiotin activity.

SUMMARY

With an initial pH of 6.5 oxybiotin is rather ineffective in causing aspartic acid synthesis and the growth of *Lactobacillus arabinosus* 17-5 is not more than one-fifth of the maximum regardless of the level of oxybiotin. When the initial pH is 7.0 or more, excellent growth results and oxybiotin is about 30 per cent as active as biotin.

BIBLIOGRAPHY

- 1. Stokes, J. L., Larsen, A., and Gunness, M., J. Biol. Chem., 167, 613 (1947).
- 2. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., J. Biol. Chem., 169, 451 (1947).
- 3. Shive, W., and Rogers, L. L., J. Biol. Chem., 169, 453 (1947).
- 4. Lichstein, H. C., and Umbreit, W. W., J. Biol. Chem., 170, 329 (1947).
- 5. Lichstein, H. C., and Umbreit, W. W., J. Biol. Chem., 170, 423 (1947).
- 6. MacLeod, P. R., and Lardy, H. A., J. Biol. Chem., 179, 733 (1949).
- Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., Science, 102, 35 (1945).
- Duschinsky, R., Dolan, L. A., Flower, D., and Rubin, S. H., Arch. Biochem., 6, 480 (1945).
- 9. Krueger, K. K., and Peterson, W. II., J. Bact., 55, 693 (1948).
- 10. Shull, G. M., and Peterson, W. H., Arch. Biochem., 18, 69 (1948).
- Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., Arch. Biochem., 7, 393 (1945).
- 12. Hofmann, K., and Winnick, T., J. Biol. Chem., 160, 449 (1945).
- 13. Axelrod, A. E., Flinn, B. C., and Hofmann, K., J. Biol. Chem., 169, 195 (1947).
- McCoy, R. H., McKibben, J. N., Axelrod, A. E., and Hofmann, K., J. Biol. Chem., 176, 1319 (1948).
- McCoy, R. H., McKibben, J. N., Axelrod, A. E., and Hofmann, K., J. Biol. Chem., 176, 1327 (1948).
- 16. Williams, V. R., and Fieger, E. A., Ind. and Eng. Chem., Anal. Ed., 17, 127 (1945).
- 17. Williams, W. L., Broquist, H. P., and Snell, E. E., J. Biol. Chem., 170, 619 (1947).
- 18. Potter, R. L., and Elvehjem, C. A., J. Biol. Chem., 172, 531 (1948).
- 19. Stokes, J. L., Larsen, A., and Gunness, M., J. Bact., 54, 219 (1947).
- 20. Lardy, H. A., Potter, R. L., and Burris, R. H., J. Biol. Chem., 179, 721 (1949).

- Winnick, T., Hofmann, K., Pilgrim, F. J., and Axelrod, A. E., J. Biol. Chem., 161, 405 (1945).
- Luckey, T. D., Moore, P. R., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 61, 97 (1946).
- Rubin, S. H., Flower, D., Rosen, F., and Drekter, L., Arch. Biochem., 8, 79 (1945).
- 24. Wyss, O., Lilly, V. G., and Leonian, L. H., Science, 99, 18 (1944).

TRIPHOSPHOPYRIDINE NUCLEOTIDE-CYTOCHROME & REDUCTASE IN LIVER

By B. L. HORECKER

(From the Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, October 20, 1949)

In yeast the reduction of ferricytochrome c by reduced triphosphopyridine nucleotide (TPNH₂) is catalyzed by a flavoprotein, cytochrome c reductase, which contains flavin mononucleotide (FMN, riboflavin phosphate) as the prosthetic group (1). In animal tissue the reduction of ferricytochrome c by TPNH₂ has not yet been reported, although the reaction with reduced diphosphopyridine nucleotide (DPNH₂) has been observed with pig heart suspensions (2) and liver extracts (3). TPN-cytochrome c reductase has now been isolated from pig liver¹ and identified as a flavoprotein with flavin-adenine dinucleotide (FAD) as the prosthetic group. The specific activity of the purified enzyme is the same as that of yeast cytochrome c reductase. In the present report the details of the isolation procedure and the properties of the purified liver enzyme are described.

Test Methods and Materials

Test System—Enzyme activity was determined essentially as previously described (1) in a test system consisting of glucose-6-phosphate, Zwischenferment, TPN, and cytochrome c, as shown in Table I. The following reactions are involved.

Glucose-6-phosphate + TPN $\xrightarrow{Zwischenferment}$ 6-phosphogluconic acid + TPNH₂ TPNH₂ + 2 ferricytochrome c $\xrightarrow{cytochrome c reductase}$ TPN + 2 ferrocytochrome c

Before addition of the cytochrome c reductase and cytochrome c, the reaction mixture was incubated for 15 minutes at room temperature to permit complete reduction of the TPN. Cytochrome c reductase activity in the Zwischenferment preparation prevented the use of larger quantities to accelerate the reduction. Corrections for this activity were made from blank determinations without added cytochrome c reductase. The rate of formation of ferrocytochrome c was followed at 549.5 m μ with the Beckman model DU spectrophotometer, with a nominal band width of 0.5 m μ . Although the reduction appeared to follow a first order course, it was nearly

¹ A preliminary report of this work was presented at the Fortieth annual meeting of the American Society of Biological Chemists at Detroit, 1949 (4).

linear in the early stages, as shown in Table I. The unit of enzyme activity was defined as the quantity which produced a density change of 1.0 per minute, calculated from the density change observed in the first 2 minutes. The specific activity is the number of units per mg. of protein.

Protein was determined by the turbidimetric method of Bücher (5). The turbidity, measured at 340 m μ with the Beckman spectrophotometer, was standardized on the basis of the dry weight and nitrogen content of a purified enzyme preparation.

Table I
Test for Cytochrome c Reductase Activity

The test system contained 0.10 cc. of glucose-6-phosphate, 25 μ m per cc.; 0.02 cc. of Zwischenferment, 4 mg. per cc.; 0.05 cc. of TPN, 1.1 μ m per cc.; 0.06 cc. of cytochrome c, 4×10^{-4} m; 0.04 cc. of cytochrome c reductase, 0.29 γ , and 1.25 cc. of 0.05 m phosphate buffer, pH 7.55. Temperature, 23-26°; length of cell, 1.0 cm.; λ = 549.5 m μ ; Beekman spectrophotometer.

Time	Density	ΔFe ⁺⁺⁺ *		
386.		μ <u>μ</u> × cc. ⁻¹ × min. ⁻¹		
0		(0.0033)†		
30	0.218			
		0.00306		
60	0.248			
		0.00256		
90	0.273			
		0.00224		
120	0.295			
		0.00204		
150	0.315			

^{*} The change in concentration of ferricytochrome c (Δ Fe⁺⁺⁺) was calculated from the equation $(d_{n+1} - d_n)/(1.96 \times 10^4)$, where d_{n+1} and d_n are successive density readings and 1.96×10^4 represents the difference in the extinction coefficients for ferro- and ferricytochrome c.

Glucose-6-phosphate—This was synthesized by the method of Fischer and Lardy (6). The final product, obtained as the Ba salt, contained 7.40 per cent P and no inorganic P; the purity based on the P content was 94 per cent. The purity was also determined by the reduction of TPN. 39.3 γ of the Ba salt of glucose-6-phosphate, containing 9.4 \times 10⁻³ μ M (micromole) of P, brought about the reduction of 8.7 \times 10⁻³ μ M of TPN. Of the total P, 93 per cent is therefore present as glucose-6-phosphate. For the enzyme assays barium was removed by addition of the calculated amount of K_2 SO₄.

Zwischenferment-Crude Zwischenferment was prepared from Anheuser-

[†] Extrapolated to zero time.

Busch brewers' yeast dried at low temperature, according to the procedure of Warburg and Christian (7). In order to obtain a product with low cytochrome c reductase content, it was necessary to study the relative activities of the two enzymes in autolysates prepared under various conditions. High temperatures appeared to favor the destruction of the cytochrome c reductase; autolysis for 6 hours at 40° was optimal for the lot of yeast used. The dry preparation obtained was about 2 per cent as active as the purified preparation described by Negelein and Haas (8).

Triphosphopyridine Nucleotide—TPN of purity 0.55 was prepared from liver by a modification of the method of Warburg, Christian, and Griese (9). The purity was determined spectrophotometrically with 6.24×10^6 sq. cm. \times mole⁻¹ as the extinction coefficient of TPNH₂ at 340 m μ (10).

Cytochrome c—This was prepared from beef heart by the procedure of Keilin and Hartree (11).

Other Substances—FAD was prepared from yeast by the method of Warburg and Christian (12). FMN was obtained from the dinucleotide by hydrolysis with nucleotide pyrophosphatase from potato (13). 0.2 mg. of FAD in 2.0 cc. of H₂O was treated with 0.01 cc. of nucleotide pyrophosphatase containing 0.22 mg. of protein per cc. (280 units per cc.). The mixture was incubated at 34° for 30 minutes, when the fluorescence had increased to a maximal value. The solution thus obtained (FMN) was stored at 0°. The protein of p-amino acid oxidase was prepared according to the procedure of Negelein and Brömel (14) through Step E. DPN was prepared by the method of Williamson and Green (15) and reduced by the method of Ohlmeyer (16). Lactic dehydrogenase was a preparation from rabbit muscle (17).

Purification of Enzyme

Preparation of Acetone Powder—Liver was obtained from freshly slaughtered pigs, sliced into thin strips, and chilled in crushed ice. 100 gm. portions were homogenized in Waring blendors with 500 cc. of acetone cooled to -10° . The residue was filtered with suction, again homogenized with cold acetone, filtered, and dried at room temperature. The powder was stored at 2° and used within 2 weeks. It was important to select liver which was pale in appearance, since dark red liver contributed substances which interfered with the ammonium sulfate and pH fractionations, greatly reducing both yield and purification.

Extraction—50 gm. of acetone powder were suspended in 750 cc. of 0.1 m Na₂HPO₄ and shaken gently for 10 minutes at 2° . The suspension was centrifuged and the supernatant, which contained most of the soluble proteins but negligible cytochrome c reductase activity, was discarded. The precipitate was vigorously homogenized in a Potter-Elvehjem homo-

genizer (18) with small additions of 0.1 M Na₂HPO₄ until about 50 cc. had been added and a smooth brown paste obtained. The thick homogenate was added to 700 cc. of 0.1 M Na₂HPO₄, mixed, and centrifuged for 5 minutes at 2500 R.P.M. The supernatant was poured off through fine gauze. About 700 cc. of a light brown suspension were obtained which could be kept for several days without loss of activity (acetone powder extract) (Table II).

Digestion with Trypsin, Ammonium Sulfate Fractionation—4.08 liters of extract from 300 gm. of acetone powder were treated with 1.32 gm. of trypsin (Wilson, 1:300) and incubated for 30 minutes at 34°. The mixture was cooled to 2° and treated with 258 gm. of ammonium sulfate per liter. After 30 minutes, the precipitate was centrifuged and discarded. The supernatant was treated with 342 gm. of ammonium sulfate per liter.

Total Specific Step units* activity units per 171. E I. Acetone powder extract (from 300 gm powder) 10,200 0.11II. Ammonium Sulfate I 8,660 1.71 III. pH ppt 5,910 6.4 IV. Calcium phosphate gel eluate 5,280 24 V. Ammonium Sulfate II 3,690 70 VI. Aluminum hydroxide gel eluate 2,630 140 VII. Repeat Steps V and VI 140-160

Table II

Preparation of Cytochrome c Reductase

The precipitate was collected on a Büchner funnel, dissolved in 600 cc. of water, and dialyzed for 18 hours against 0.04 m sodium acetate. The pH of the acetate solution was 6.8 to 7.2, owing to residual acid in the demineralized water used in its preparation. The dialyzed solution lost about 10 per cent of its activity when stored overnight at 2° (Ammonium Sulfate I, Table II).

pH Precipitation—Since the pH range for optimal precipitation of the enzyme in the dialyzed solution varied considerably from preparation to preparation, it was necessary to make a small pilot run in each case. With 30 cc. (Ammonium Sulfate I), fractions were collected at pH 5.3, 5.1, 4.9, and 4.7. For the preparation described here most of the enzyme was precipitated between pH 5.3 and 4.9. The dialyzed solution (540 cc.) was brought to pH 5.26 with 39 cc. of 0.2 n acetic acid and after 5 minutes at 2° the solution was centrifuged and the precipitate discarded. The

^{*} Density change per minute.

supernatant solution was acidified to pH 4.87 with 53 cc. of 0.2 n acetic acid, again kept 5 minutes, and then centrifuged. This precipitate was dissolved in water with the aid of 11.4 cc. of 0.2 n NH₄OH; the volume was 153 cc. and the pH about 8.0. To prepare the enzyme for the calcium phosphate gel step, it was precipitated with 95 gm. of ammonium sulfate. The precipitate was dissolved in water and adjusted to about pH 8.0 with 4.0 cc. of 2 n NH₄OH (pH precipitate, 150 cc.).

Calcium Phosphate Gel Adsorption and Elution—Calcium phosphate gel (19), aged 3 to 6 months, was added until the supernatant was pale pink, with all the yellow components adsorbed. 150 cc. of the pH precipitate required 220 cc. of the gel (dry weight 8.8 mg. per cc.). The gel was centrifuged and was washed two times in the centrifuge with 60 cc. of 0.01 m phosphate buffer, pH 7.4, and the enzyme was eluted four times with 60 cc. of 0.1 m K₂HPO₄ (calcium phosphate gel eluate).

Lyophilization—240 cc. of eluate were precipitated with 167 gm. of ammonium sulfate. The precipitate was dissolved in 30 cc. of 0.15 m pyrophosphate buffer, pH 7.6, and lyophilized. 3.0 gm. of powder were obtained containing 150 mg. of protein. The powder was stable for several months at 3° in vacuo.

Alkaline Ammonium Sulfate Fractionation—Since the concentration of ammonium sulfate at which optimal precipitation occurred was somewhat variable, a number of fractions were collected, tested separately, and the best ones pooled for the subsequent steps. 3.0 gm. of powder were dissolved in 200 cc. of H₂O and treated with 76 gm. of ammonium sulfate and 4.0 cc. of concentrated ammonia water to bring the pH to about 8.0. The precipitate was collected by centrifugation and dissolved in 15 cc. of water. The supernatant was treated with 7.7 gm. of ammonium sulfate, the precipitate was collected and dissolved, and the supernatant again treated with 7.7 gm. of ammonium sulfate. In this way a total of six fractions was collected of which the third, fourth, and fifth were combined (Ammonium Sulfate II, 48 cc.).

Aluminum Hydroxide Gel Adsorption and Elution—The combined ammonium sulfate fractions were diluted with 40 cc. of water and treated with 40 cc. of aluminum hydroxide gel $C\gamma$ (20) aged 6 months or longer (dry weight 11.4 mg. per cc.). The gel was centrifuged and washed twice with 10 cc. portions of 0.025 m phosphate buffer, pH 7.2, and the enzyme was eluted six times with 10 cc. portions of 0.1 m phosphate buffer, pH 7.2 (aluminum hydroxide gel eluate).

Further Purification—A further small purification was usually accomplished by repetition of the last two operations, alkaline ammonium sulfate and aluminum hydroxide gel adsorption; the final specific activity was usually 140 to 160 units per mg. The purified enzyme was unstable

in solution and slowly lost activity when frozen and stored at -20° , or when lyophilized as described above. In 20 minutes at 34° one-third of the activity was destroyed.

Absorption Spectrum

Solutions of purified cytochrome c reductase with specific activity of 150 to 160 units per mg. are bright yellow and show the characteristic

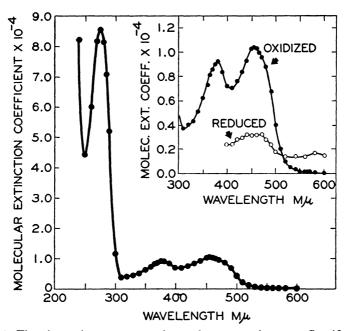


Fig. 1. The absorption spectrum of cytochrome c reductase. Specific activity = 162 units per mg. The extinction coefficients were based on a value of 1.04×10^6 sq. cm. \times mole⁻¹ at 455 m μ . The reduced spectrum was obtained by the addition of 0.20 mg. of Zwischenferment, 1.25 μ m of glucose-6-phosphate, and 0.03 μ m of TPN in a total volume of 0.3 cc. In the insert the portion between 300 and 400 m μ is enlarged to show the band details.

flavoprotein absorption spectrum (Fig. 1), with maxima at 275, 380, and 455 m μ . With less pure preparations a strong hemin band at about 410 m μ completely obscures the flavoprotein spectrum; this component was removed in the last purification step. On reduction with excess TPNH₂ the absorption at 455 m μ was largely but not entirely removed. The purity of the enzyme preparation may be estimated from the content of reducible flavoprotein. The change in density observed after addition

of excess TPN, Zwischenferment, and glucose-6-phosphate in the experiment described in Fig. 1 was 0.071. With the assumption that the extinction coefficient for flavoproteins at 455 m μ is 1.04 \times 10⁶ sq. cm. \times mole⁻¹ (1, 21, 22), the concentration of cytochrome c reductase was 6.8 \times 10⁻⁸ mole per cc. The total protein present was 4.6 \times 10⁻⁴ gm. per cc., from which the molecular weight was calculated to be 68,000. This result is in agreement with molecular weights of 72,000, 65,000, and 78,000 obtained from the flavin content for "old" yellow enzyme (21), "new" yellow enzyme (22), and yeast cytochrome c reductase (1), respectively, and of 83,000 and 78,000 for "old" yellow enzyme determined by ultracentrifugation (23, 24). Since on this basis cytochrome c reductase accounted for practically all of the protein present, the residual absorption after reduction was due to a contaminant of high specific absorption.

Prosthetic Group

Splitting and Resynthesis—Cytochrome c reductase was split by treat¹ ment with acid in the presence of ammonium sulfate as described by Warburg and Christian (12). 4.0 cc. of aluminum hydroxide gel eluate (1.80 mg. of protein) were treated with 0.9 gm. of ammonium sulfate and 0.4 cc. of 1.0 n H₂SO₄ containing 0.22 gm. of ammonium sulfate per cc. After 10 minutes at 0°, the precipitate was centrifuged and dissolved in 2.0 cc. of 0.1 m phosphate buffer, pH 7.0. The supernatant after neutralization with 0.4 cc. of 0.2 n NH₄OH had a volume of 6.5 cc. These solutions will be referred to as Protein R and Flavin R, respectively.

The reactivation of the split protein was studied with FAD and FMN prepared as described in the first section, with results as shown in Fig. 2.2

The residual activity of Protein R was increased by either FAD or FMN and the mononucleotide was considerably more effective than the dinucleotide. Although the data do not permit a precise determination of the dissociation constants, it is apparent that half reactivation was obtained with FMN at about 1×10^{-8} m and with FAD at about 2×10^{-8} m.

Effect of Nucleotide Pyrophosphatase on Cytochrome c Reductase Flavin—While the results of the preceding section do not permit any conclusion as to the identity of the cytochrome c reductase prosthetic group, the experiments described below identify the flavin obtained from cytochrome c reductase as FAD.

Mixtures of FAD and FMN can be analyzed fluorometrically by the

² With a synthetic sample of FMN kindly furnished by Dr. C. W. Sondern of the White Laboratories, Inc., Newark, New Jersey, reactivation of cytochrome c reductase protein was about equal to that given with FMN prepared by the hydrolysis of FAD.

method of Burch, Bessey, and Lowry (25), based on the increase in fluorescence which accompanies the splitting of FAD to FMN and adenylic acid. The results with Flavin R are compared with those obtained with FAD in Table IV. Since the fluorescence of FAD is 0.14 times that of FMN, the per cent of FAD in a mixture is given by the equation

% FAD =
$$\frac{f_2 - f_1}{0.86 f_2} \times 100$$
 (1)

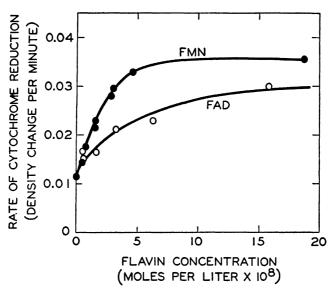


Fig. 2. The reactivation of cytochrome c reductase protein by flavins. 0.05 cc. of Protein R was incubated for 10 minutes at 0° with flavin in 0.01 m phosphate buffer in a total volume of 0.8 cc. The activity was tested as in Table I, with 0.04 cc. of incubation mixture. The flavin concentrations are the final concentrations in the test.

where f_1 and f_2 are the fluorometer readings before and after hydrolysis of the flavin mixture. In the experiment in Table III, nucleotide pyrophosphatase was used to hydrolyze the dinucleotide. As shown in Table III, all the flavin in Flavin R is accounted for as FAD.

In Table IV is shown the effect of hydrolysis of FAD and Flavin R on the reactivation of Protein R. The activities before and after hydrolysis were compared with the reactivation obtained with an excess of FMN. As would be expected from Fig. 2, FAD was more effective after hydrolysis. A similar increase in activity was observed with Flavin R. In the last line of Table IV is included an experiment with intact cytochrome c reductase.

In agreement with results obtained with p-amino acid oxidase (13), the bound flavin was not attacked by nucleotide pyrophosphatase under conditions which resulted in complete hydrolysis of the free dinucleotide.

Activity of Cytochrome c Reductase Flavin in D-Amino Acid Oxidase Test— Further evidence for the identity of the flavin of cytochrome c reductase

Table III Fluorometric Analysis of Cytochrome c Reductase Flavin

 $4.7 \times 10^{-4} \,\mu\text{M}$ of flavin was incubated for 30 minutes at 34° with 10 units of nucleotide pyrophosphatase in 1.0 cc. of 0.05 M phosphate buffer, pH 7.4. The control solution (before hydrolysis) was incubated without nucleotide pyrophosphatase. Galvanometer readings.

	Flavin R	FAD
Before hydrolysis (f_1)	5.0	11.0
After hydrolysis (f_2)	32.5	45.5
% FAD*.	98	93

^{*}Calculated from Equation 1.

Table IV Flavin Activity with Cytochrome c Reductase Protein

The flavins were incubated for 30 minutes at 34°, with 10 units of potato nucleotide pyrophosphatase in 0.75 cc. of 0.01 m phosphate buffer, pH 7.2. After incubation the solutions were cooled, 0 05 cc. of Protein R was added, and the incubation was continued for 15 minutes at 0°. Control (before splitting) were incubated without nucleotide pyrophosphatase. Tests were made with 0.04 cc. of the incubation mixture as in Table I.

	Before h	Before hydrolysis		After hydrolysis	
Flavin	Specific activity	Per cent reacti- vation	Specific activity	Per cent reacti- vation	
	units per mg.		units per mg.		
None	0 009	0			
FMN (5 7 μ M \times 10 ⁻⁸)	0 031	100			
FAD $(3.8 \mu \text{M} \times 10^{-4})$	0 016	32	0 029	91	
Flavin R $(3.7 \mu \text{M} \times 10^{-4})$	0 020	50	0.032	105	
Cytochrome c reductase	0 020		0.022]	

was provided by its activity with the protein of p-amino acid oxidase. The rate of production of pyruvate from p-alanine was measured by the oxidation of DPNH₂ in the presence of lactic dehydrogenase. The test system contained 33 μ m of pl-alanine, 0.10 μ m of DPNH₂, 22 γ of lactic dehydrogenase, and 56 γ of amino acid oxidase (Protein E) in 0.03 m pyrophosphate buffer, pH 8.3, in a final volume of 1.6 cc. The oxidation

of DPNH₂ was measured with the Beckman spectrophotometer at 340 m μ . The rate of oxidation of DPNH₂ was not linear, but increased as the oxidation progressed. Reproducible results were obtained, however, by measuring the time required for the density to change between two defined values, in this case 0.325 and 0.225. The reciprocal of the time is a measure of the FAD content. The concentration of FAD required for half activation of p-amino acid oxidase protein by this method agreed with that obtained by the manometric technique.

Solutions of cytochrome c reductase were analyzed for their FAD content by this procedure after the flavin was liberated by heating at 100° for 5 minutes. The results are shown in Table V. In the case of Preparation II, the one used in the determination of the molecular weight (Fig. 1), there is excellent agreement between FAD and flavoprotein content, the values in both cases being about 70 per cent of the total light

	FAD Content of Cytochrome c Reductuse						
Preparation No.	Total flavin calculated from absorption at 455 mm	Flavin calculated from reduction with TPNH:	FAD*				
attended to the second	μ M per cc.	µм per cc.	μM per cc.				
I	0.0082		0.0053				
II	0.0088	0.0068	0.0063				
III	0.0035		0.0028				

TABLE V
FAD Content of Cytochrome c Reductase

absorption at 455 m μ . With two other preparations of cytochrome c reductase, the FAD content accounted for 65 and 80 per cent of the total absorption at 455 m μ . These results are consistent with the conclusion that the residual absorption after reduction with TPNH₂ is due to a contaminant of high specific absorption and that FAD is the prosthetic group of liver cytochrome c reductase.

Activity of Liver Cytochrome c Reductase

Turnover Number—From the data in Table I the turnover number for liver cytochrome c reductase is 1140 moles of cytochrome c per mole of flavoprotein per minute, with the molecular weight taken to be 68,000. For the yeast enzyme under almost identical test conditions, the turnover number was calculated to be 1300 (molecular weight = 78,000). Thus, despite different prosthetic groups, the catalytic activities of the yeast and liver enzymes are nearly the same.

The reaction of liver cytochrome c reductase with oxygen is less than 2 per cent as fast as the reaction with cytochrome c.

^{*} Determined in amino acid oxidase test.

Under the conditions of the test the rate of cytochrome c reduction was independent of both TPN and cytochrome c concentration. Although the reduction appears to follow a first order course with respect to ferricytochrome c, this was not confirmed by variation of the total cytochrome c concentration, since an increase of 6-fold resulted in the same initial rate of reduction. The reaction was thus zero order with respect to cytochrome c and the decline in rate with time must be attributed to some other factor. The substrate optima for both TPN and cytochrome c are at concentrations below 10^{-5} M.

Association of Cytochrome c Reductase with Liver Particles—Cytochrome c reductase activity in the acetone powder extract is associated with insoluble particles. In this form the activity is quantitatively precipitated by ammonium sulfate below 40 per cent saturation. As a result of tryptic digestion the milky suspension becomes darker and more opalescent, the protein content falls by one-third to one-half, and 90 per cent saturation with ammonium sulfate is required for complete precipitation.

Homogenates of fresh liver, when fractionated according to the procedure of Hogeboom, Schneider, and Pallade (26), have cytochrome c reductase activity in the mitochondrial fraction. The specific activity of these particles is the same as that of the acetone powder extract.

The content of cytochrome c reductase in liver is comparable with that observed in ale yeast. 300 gm. of acetone powder derived from about 3 kilos of fresh liver yielded about 65 mg. of enzyme. From 300 gm. of dried ale yeast about 180 mg. of enzyme were obtained.

The activity of DPN-cytochrome c reductase in crude pig liver extracts is about 20 times that of the TPN enzyme. This activity is almost completely absent after the first ammonium sulfate fractionation.

Optimal pH—The optimal pH for liver cytochrome c reductase is shown in Fig. 3. There is a marked fall in activity above pH 8.4 and below pH 7.5, with negligible activity remaining at pH 6.0. From the data in Fig. 3 it is evident that there is no requirement for added phosphate in the reaction, since rates obtained in the absence of phosphate agree with those obtained in phosphate buffer. 2,4-Dinitrophenol at a concentration of 1.3×10^{-4} M did not affect the activity.

DISCUSSION

Ever since the isolation of the "old" yellow enzyme by Warburg and Christian (7), flavin enzymes have been assigned an important rôle in the oxidation of the pyridine nucleotides. There was some doubt, however, as to the requirement for accessory enzymes which would link them to

^{*} Unpublished observations by L. A. Heppel.

cytochrome c, since none of the early flavoproteins, such as the "old" yellow enzyme, "new" yellow enzyme (22), or heart flavoprotein (27), react with cytochrome c or with oxygen at rates consistent with physiological requirements for respiration. With the isolation of cytochrome c reductase from yeast (1), it was demonstrated that a flavoprotein could react directly with cytochrome c at a rapid and physiological rate. No other intermediate was found nor was it necessary to postulate one. In the present paper a similar enzyme from animal tissue is described. While the liver enzyme differs from that isolated from yeast in that FAD appears to be the prosthetic group, it is of interest that with FMN a synthetic

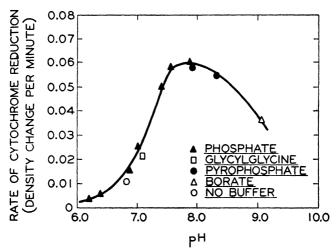


Fig. 3. Activity of cytochrome c reductase as a function of pH. The buffers were all 0.04 m. Test as in Table I with 0.7 mg. of cytochrome c reductase; specific activity = 54.

enzyme is obtained having a higher activity. With both "old" yellow enzyme (28) and yeast cytochrome c reductase synthetic enzymes could be prepared in which the FMN is replaced by FAD. In both cases the "FAD enzyme" is only one-third to two-thirds as active as the "FMN enzyme."

The author is indebted to Dr. L. A. Heppel for DPNH₂ and amino acid oxidase protein, to Dr. A. Kornberg for preparations of nucleotide pyrophosphatase and lactic dehydrogenase, and to Dr. A. Schrecker for FAD. Thanks are also due to Miss Pauline Smyrniotis for valuable technical assistance.

Unpublished observations.

SUMMARY

- 1. TPN-cytochrome c reductase has been isolated in essentially pure form from pig liver acetone powder.
- 2. The spectrum is that of a flavoprotein, with maxima at 275, 380, and 455 m μ . The molecular weight calculated from the flavin content is 68,000.
- 3. The enzyme can be split into protein and flavin fractions. The protein is reactivated by either flavin mononucleotide (FMN) or flavinadenine dinucleotide (FAD).
- 4. From its hydrolysis with nucleotide pyrophosphatase and activity in the p-amino acid oxidase system, the flavin of cytochrome c reductase appears to be FAD.
- 5. The specific activity and concentration of liver cytochrome reductase are comparable with those of cytochrome c reductase in yeast.

BIBLIOGRAPHY

- 1. Haas, E., Horecker, B. L., and Hogness, T. R., J. Biol. Chem., 136, 747 (1940).
- 2. Lockhart, E. E., and Potter, V. R., J. Biol. Chem., 137, 1 (1941).
- 3. Heppel, L. A., Federation Proc., 8, 205 (1949).
- 4. Horecker, B. L., Federation Proc., 8, 207 (1949).
- 5. Bücher, T., Biochem. et biophys. acta, 1, 292 (1947).
- 6. Fischer, H. O. L., and Lardy, H. A., J. Biol. Chem., 164, 513 (1946).
- 7. Warburg, O., and Christian, W., Biochem. Z., 254, 438 (1932).
- 8. Negelein, E., and Haas, E., Biochem. Z., 282, 206 (1935).
- 9. Warburg, O., Christian, W., and Griese, A., Biochem. Z., 282, 157 (1935).
- 10. Horecker, B. L., and Kornberg, A., J. Biol. Chem., 175, 385 (1948).
- 11. Keilin, D., and Hartree, E. F., Biochem. J., 39, 289 (1945).
- 12. Warburg, O., and Christian, W., Biochem. Z., 298, 150 (1938).
- 13. Kornberg, A., and Pricer, W. E., Jr., J. Biol. Chem., 182, 763 (1950).
- 14. Negelein, E., and Bromel, H., Biochem. Z., 300, 225 (1939).
- 15. Williamson, S., and Green, D. E., J. Biol. Chem., 135, 345 (1940).
- 16. Ohlmeyer, P., Biochem. Z., 297, 66 (1938).
- 17. Kornberg, A., J. Biol. Chem., 182, 805 (1950).
- 18. Potter, V. R., and Elvehjem, C. A., J. Biol. Chem., 114, 495 (1936).
- 19. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. London, Series B, 124, 397 (1938).
- 20. Willstätter, R., and Kraut, H., Ber. chem. Ges., 56, 1117 (1923).
- 21. Theorell, H., Biochem. Z., 278, 263 (1935).
- 22. Haas, E., Biochem. Z., 298, 378 (1938).
- 23. Keckwick, R. A., and Pedersen, K. O., Biochem. J., 30, 2201 (1936).
- 24. Polson, A. G., in Svedberg, T., Nature, 139, 1060 (1937).
- 25. Burch, H. B., Bessey, O. A., and Lowry, O. H., J. Biol. Chem., 175, 457 (1948).
- Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., J. Biol. Chem., 172, 619 (1948).
- 27. Straub, F. B., Biochem. J., 33, 787 (1939).
- 28. Warburg, O., and Christian, W., Biochem. Z., 298, 368 (1938).

THE LINKAGE OF THE AMINO GROUP IN HEPARIN*

By J. ERIK JORPES, HARRY BOSTRÖM, AND VIKTOR MUTT

(From the Chemistry Department of the Caroline Institute and the Insulin Laboratory of the Vitrum Company, Stockholm, Sweden)

(Received for publication, August 31, 1949)

Alleged Acetyl Content of Heparin

Many erroneous assumptions have been made from time to time as to the chemical nature and the composition of heparin. To these we must now add the assumption (1-6) that heparin contains an acetyl group, which at one time seemed fairly well justified. As pointed out by Fürth and coworkers (7), the amino sugar is monoacetylated in all the known mucopolysaccharides and in chitin. Thus, acetic acid occurs in chondroit-insulfuric acid (8), in the hyaluronic acid of Meyer (3), in the immunopolysaccharide of type I pneumococci (9), and in the blood group substances (10, 11). The first analytical findings on heparin also supported this view.

The analytical methods applied, however, soon proved somewhat unreliable. The Kuhn-Roth (12) alkaline hydrolysis in methyl alcohol, applied in the first communication (1), later proved, when applied to chondroitinsulfuric acid (2), to liberate a large excess of acid, as found simultaneously by Friedrich and Sternberg (13). When, on the contrary, the toluenesulfonic acid was used for hydrolysis, either as recommended by Friedrich and Rapoport (14) or as modified by Friedrich and Sternberg, no acetic acid whatsoever was found in heparin.

Taking into account the extreme slowness with which reducing groups are liberated on acid hydrolysis of heparin, Jorpes and Bergström, who unfortunately did not follow the liberation of the amino group quantitatively, thought that strong hydrolytic agents were necessary, such as 10 per cent (by volume) of sulfuric acid. In fact, acetic acid was also identified by them as silver salt in the distillate after acid hydrolysis.

In the meantime a negative finding was made by Masamune, Suzuki, and Kondoh (15) and by Wolfrom and coworkers (16). The latter authors applied both the toluenesulfonic acid method and the chromic acid oxidation method for the determination of the CH₃C group.

The findings of the Japanese workers and of Wolfrom and coworkers necessitated a reinvestigation of the question with more reliable methods.

^{*} This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm.

Nature and Amount of Acid Distilled after Acid Hydrolysis of Heparin—At first, the nature of the volatile fatty acid formed on submitting the heparin and related substances to hydrolysis with 10 per cent (by volume) of sulfuric acid during 4 to 48 hours was determined qualitatively by means of the distillation constant, as described by Clark ((17) p. 85). The values for the distillation constant found were formic acid 20.8 ± 0.22 , acetic acid 35.6 ± 0.56 , and propionic acid 55.8 ± 0.50 .

Only acetic acid was liberated from chondroitinsulfuric acid and from a water-soluble fraction of the barium salt of crude heparin. From the

Table I

Acetic Acid Found When Heparin and Related Substances Were Submitted to Acid Hydrolysis

	Hydr	olysis	Acid found				
	Per cent		Before tre		After treatment with HgO		
	(by vol- ume) of sulfuric acid	Time	Per cent*	Distil- lation con- stant	Per cent†	Distil- lation con- stant	
		hrs.	Ministration Printer	-			
Heparin (Na salt),	10	24	3.87	29.3	0.39	36.9	
12.97% sulfur	25	48	13.85		0.47	36.9	
	25	24			0.52	36.7	
	25	48			0.47	37.9	
Chondroitinsulfuric	10	24	7.3	38.8	5 5 (Theoretical 12.1)	34.0	
acid (Ca salt)	25	36			4.8	36.2	
Chitin	25	48			21.5 (Theoretical 26.8)	38.0	
	25	20			7.8	36.5	
	10	48			4.1	36.4	

^{*} Calculated as acetic acid.

less soluble barium salt of heparin a mixture of acetic acid and formic acid was obtained. Starch, glucose, galactose, and glucosamine yielded only formic acid.

The next step was to remove the formic acid through oxidation with mercuric oxide (see Clark ((17) p. 89)) and to determine the remaining acetic acid quantitatively by titration. The results are given in Table I.

When submitted to hydrolysis with sulfuric acid, chondroitinsulfuric acid and chitin thus gave considerable amounts of acetic acid. The heparin sample gave about a tenth of the theoretical figure. The small amount of acetic acid found could derive from the polysaccharide related to heparin,

[†] No correction is made for the loss of acetic acid during the treatment with HgO. In treating mixtures of acetic and formic acids in the same way, the loss varied between 20 and 30 per cent of the acetic acid present.

which follows the heparin and can be separated from it as a more soluble barium salt.

Content of Acetic Acid As Determined According to Lemieux and Purves—Since the chromic acid method of Kuhn and l'Orsa for the determination of terminal methyl groups in sugars, as modified by Lemieux and Purves (18) for the determination of acetic acid, gave the best results on analyzing chondroitinsulfuric acid, we applied this method to our samples. The results are given in Table II.

The chromic acid method of Lemieux and Purves showed, as did the hydrolysis with acids, a decreasing content of acetic acid with increasing purity of the heparin samples. Almost correct values were found for chondroitinsulfuric acid. In an easily soluble barium salt of the heparin polysaccharide with 7.25 per cent sulfur, 3.05 and 2.59 per cent of acetic

Table II

Quantitative Determination of Acetic Acid According to Lemieux and Purves

	Sulfur	Nitrogen	Acetic acid Per cent dry substance	
	Per cent	Per cent		
	dry substance	dry substance	Found	Theo- retical
Chondroitinsulfuric acid (Ca salt).		1.99	11.5 11.9 11.4	12.1
Heparin (easily soluble barium salt).	7.25	1.86	$\frac{3.05}{2.59}$	
" (insoluble barium salt)	10.31	2.05	0.92 0.86 0.86	

acid were found. The low content of acetic acid, less than 1 per cent, found on hydrolyzing with acid a sodium salt of the purer heparin containing 12.97 per cent of sulfur, and about 0.9 per cent found with the chromic acid method on analyzing a barium salt with 10.31 per cent of sulfur, certainly derives from an admixture of impurities. The more soluble barium salts, obtained by fractionating the crude heparin polysaccharide, contain acetic acid, as shown by Jorpes and Gardell (19).

Determination of Acetic Acid by Means of Transesterification—We also tried the transesterification method of Stuart (20), using an apparatus of the same construction as that used by him, following his description of the method exactly. The formation of volatile ethyl acetate makes this method highly specific and very useful for our purpose. We also followed the liberation of the amino group. The results are given in Table III.

With this method also, 7 per cent of the theoretical figure was obtained for the acetic acid content of chondroitinsulfuric acid after 4 hours distillation. During distillation there was a fairly good correlation between the NH₂ nitrogen and the acetyl liberated. On the other hand, about only 10 per cent of the theoretical figure for the acetyl content of heparin was obtained after 5 hours distillation, although the amino group was totally free after 2 hours.

Linkage of Amino Group

Heparin thus constitutes an exception to the rule that the amino sugars of the animal polysaccharides are monoacetylated. The linkage of the amino group must be explained in another way. There are no NCH₃ groups in heparin (16). Masamune and coworkers suggested an anhydride linkage,—C -NHSO₂—OC—to one of the sulfuric acid groups. This pos-

Table III

Liberation of NH₂ Nitrogen and Acetic Acid from Chondroitinsulfuric Acid and
Heparin in Transesterification Process, As Modified by Stuart

A DESCRIPTION OF THE PROPERTY AND ADDRESS OF THE PROPERTY ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY AND ADDRESS OF THE PROPERTY ADDRESS	THE STATE OF THE S				
	1	Time	NH ₂	Acetic	acid
		- I mie	gen	Found	Theo- retical
		hrs	per cent	per cent	per cent
Chondroitmsulfuric acid (Ba salt)		2	1.44	4.08	
		3	1.70	5.78	12.1
		4	2.03	8.40	
Heparin, Ba salt (9 25% sulfur)		2	1.76	0.28	
		5	1.76	0.69	

sibility is excluded because all the ester sulfuric acid of heparin is ionized and can be neutralized with alkali. As shown by Wilander (21), the free acid of heparin, prepared through electrodialysis, consumes 4 equivalents of alkali, 3 within the range below pH 3 corresponding to the three sulfuric acid groups and the 4th between pH 3 and 7 corresponding to the carboxyl group. The amino group was not free, as could be shown by the lack of buffering capacity between pH 7 and 10. The same was found by Wolfrom and coworkers (16). The Ba:S ratio of their acid barium salt was 1:2 and the carboxyl group could be titrated quantitatively.

Wolfrom and coworkers (16) discussed the different possibilities which could be considered and found a linkage to the sugar aldehyde group possible. Such a linkage is, however, highly improbable because of the extremely slow liberation of reducing groups during acid hydrolysis of heparin. When boiled with 7.5 per cent (by volume) of sulfuric acid for 5 to 10

minutes, heparin liberates reducing groups, calculated as glucose, corresponding to only 1 to 2 per cent of the organic material (2). In fact this principle has been used later (19) to differentiate heparin and the polysaccharide related to it from hyaluronic acid and chondroitinsulfuric acid, which are both easily hydrolyzed. Wolfrom and McNeely (22) made the same observation regarding the absence of reducing groups. In our experiments the amino group of heparin was liberated through boiling with 0.04 N hydrochloric acid for about 3 hours without any reducing capacity being found. Nor were any volatile acids or aldehydes liberated. Consequently, the aldehyde group of the uronic acid moiety of heparin cannot be linked to the amino group.

Sulfuric Acid Bound to Amino Group

Wolfrom and McNeely (22) paid due attention to the possibility of a sulfate group being bound to the amino group. They could not, however,

Table IV

Relative Amounts of NH₂ Nitrogen and Sulfuric Acid Split Off from Heparin at 100° in 0.04 N HCl

Time	NH2 nitrogen	-SO ₂ OH
mis.	m.eq.	m.eq.
7	16.4	Trace
15	27.8	14.1
45	68.5	76.5
60	74.5	94.5

find any correspondence in the amounts of sulfuric acid and amino nitrogen liberated during acid hydrolysis, the former being only 50 per cent the expected value. As is seen in Table IV, we also found no strict relationship, the amount of sulfuric acid found in the beginning being too low. After half an hour it tended to exceed the amount of amino nitrogen.

The first opalescence of barium sulfate appeared when about 12 per cent of the nitrogen in heparin was set free as NH₂. At this stage, and even later, it was not possible to separate the barium sulfate quantitatively. The heparin polysaccharide kept it in a colloidal state. It could first be separated when the heparin molecule had been depolymerized. We made use of the greater stability of the nitrogen linkage in alkaline solution to reach this goal.

Whereas the nitrogen of heparin is set free as NH₂ nitrogen in 0.04 N HCl at 100° for about 3 hours, no NH₂ nitrogen, or very little, is set free on boiling in normal sodium hydroxide solution for 1 hour. Ester sulfates are liberated corresponding to about 25 per cent the total. 75 per cent of

the anticoagulant activity is destroyed in 5 minutes and practically all in 1 hour. The appearance of the amino group in alkaline solution therefore bears no relationship to the disappearance of the anticoagulant activity.

The sodium salt obtained after boiling heparin in N NaOH for 1 hour contained 9.5 per cent sulfur, 2.9 per cent total nitrogen (calculated about 2 per cent), and 0.31 per cent NH₂ nitrogen. At least 85 per cent the nitrogen of heparin was still bound in the original linkage. The starting material was a sodium salt with 12.2 per cent sulfur, 2.14 per cent nitrogen, and 0.24 per cent amino nitrogen. After alkaline hydrolysis the solution was

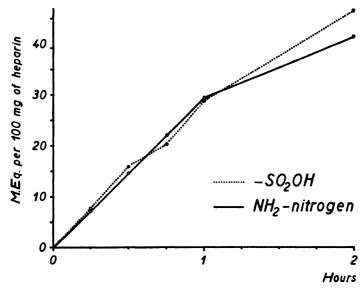


Fig. 1. Relative amounts of amino nitrogen and sulfur split off when a 2 per cent solution of the alkali-treated heparin was boiled in 0.04 n HCl. The amount of nitrogen set free as NH₂ nitrogen during 2 hours corresponds to 30 per cent of the calculated nitrogen content of the alkali-treated heparin.

cooled and neutralized with HCl, dialyzed against distilled water, concentrated, and treated with alcohol. When this preparation was submitted to acid hydrolysis in 0.04 n HCl, exactly equivalent amounts of sulfate and NH₂ nitrogen were liberated, as is seen in Fig. 1.

The acid hydrolysis was repeated four times with three different samples of the depolymerized, partly hydrolyzed heparin.

In the hydrolysis shown in Fig. 1, 21 per cent of the nitrogen calculated to belong to the heparin was set free as NH₂ nitrogen in 1 hour and 30 per cent in 2 hours. In another hydrolysis, 27 per cent of the nitrogen was liberated in 30 minutes and 35 per cent in 1 hour. After 30 minutes the

amounts were 39 m.eq. of NH₂ nitrogen and 34 m.eq. of —SO₂OH and, after 1 hour, 49 m.eq. of NH₂ nitrogen and 46 m.eq. of —SO₂OH.

In two other hydrolyses a smaller effect was obtained because the solution, 3.5 per cent, was more concentrated, giving a weaker acidity, and the volume was a little larger. Here the first trace of NH₂ nitrogen appeared after 15 minutes simultaneously with traces of free sulfates. After 30, 45, and 60 minutes identical figures were obtained for the two entities, although only 5 per cent of the nitrogen was liberated. On prolonged acid hydrolysis the figures for the sulfur equivalent slightly exceeded those for the NH₂ nitrogen equivalent, at 2 hours by 10 per cent and at 8 hours by 20 per cent, at which time 60 per cent of the nitrogen was set free as amino nitrogen.

The figures for the amino nitrogen are correct within 2 to 3 per cent. The double analysis for sulfur usually checks within the same range, but in this case the variations were greater, *i.e.* 5 to 10 per cent.

DISCUSSION

Acetic acid cannot occur in heparin, as is shown by different methods of analysis, which gave good yields of acetic acid when ordinary mucopoly-saccharides were analyzed. Nor can the amino group be involved in any linkage to the aldehyde group of the glucuronic acid or of the glucosamine itself because no aldehyde groups appear simultaneously with the NH₂ nitrogen. A linkage to sulfate groups must therefore be considered.

In fact, free sulfuric acid does under certain conditions appear simultaneously with the amino nitrogen with exactly the same speed and in equimolecular amounts. This could, of course, be only coincidental, but the similarity in the rates of hydrolysis is so close that a linkage between the two groups is highly probable. Consequently, the amino group should occur in a —NHSO₂OH linkage as a substituted amidosulfuric or sulfamic acid. The properties of these compounds also closely resemble those of heparin. The amidosulfuric acid, first isolated by Berglund in Lund, Sweden, 1875 (23–25), is very labile in acid solution and fairly resistant in the presence of alkali.

This is also true of the amino linkage in heparin. It is disrupted at 100° in $0.04~\mathrm{N}$ HCl in 3 hours, whereas it resists heating in NaOH for 1 hour. When it breaks up in acid solution, equimolecular amounts of sulfuric acid and NH₂ groups are set free, at least until 30 per cent of the nitrogen linkage is hydrolyzed.

Consequently, in heparin, sulfuric acid replaces the acetic acid of the other mucopolysaccharides. The theory that sulfuric acid constitutes the prosthetic group of heparin is thereby still further substantiated.

SUMMARY

In accordance with earlier findings of Wolfrom et al., purified heparin was found to contain only a trace of acetic acid considered to arise from impurities, when analyzed by different methods which, when applied to mucopolysaccharides, give good yields.

The amino group of glucosamine in heparin is not linked to any aldehyde group.

When heparin is boiled in N NaOH, it depolymerizes and loses 75 per cent its anticoagulant activity in 5 minutes. Very little NH₂ nitrogen appears even after 1 hour. If this heparin is subsequently submitted to weak acid hydrolysis in 0.04 N IICl, free sulfuric acid appears at the same time and with the same rate as the NH₂ groups.

Consequently, in heparin a sulfuric acid group is bound to the amino group with a substituted amidosulfuric acid, —NHSO₂OH, as the most probable type of linkage.

The authors are greatly indebted to Mr. Gunnar Lindén, Apoteksvarucentralen Vitrum, for his assistance during the performance of the work.

BIBLIOGRAPHY

- 1. Jorpes, E., Naturwissenschaften, 23, 196 (1935).
- 2. Jorpes, E., and Bergstrom, S., J. Biol. Chem., 118, 447 (1937).
- Meyer, K., and Smyth, E. M., Cold Spring Harbor Symposia Quant. Biol., 6, 97 (1938).
- 4. Reinert, M., and Winterstein, A., Arch. internat. pharmacod., 62, 47 (1939).
- 5. Charles, A. F., and Todd, A. R., Biochem. J., 34, 112 (1940).
- Jorpes, E., Heparin in the treatment of thrombosis, London and New York, 2nd edition (1946).
- 7. Fürth, O., Hermann, H., and Scholtz, R., Biochem. Z., 271, 395 (1934).
- 8. Mörner, C. T., Skand. Arch. Physiol., 1, 210 (1889-90).
- 9. Heidelberger, M., Avery, O. T., and Goebel, W., J. Exp. Med., 42, 727 (1925).
- 10. Landsteiner, K., J. Exp. Med., 63, 185 (1936).
- 11. Freudenberg, K., Westphal, O., and Groenewoud, Naturwissenschaften, 24, 522 (1936).
- 12. Kuhn, R., and Roth, H., Ber. chem. Ges., 66, 1274 (1933).
- 13. Friedrich, A., and Sternberg, H., Biochem. Z., 286, 20 (1936).
- 14. Friedrich, A., and Rapoport, S., Biochem. Z., 251, 432 (1932).
- 15. Masamune, H., Suzuki, M., and Kondoh, Y., J. Biochem., Japan, 31, 343 (1940).
- Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H., and McLean, J., J. Am. Chem. Soc., 65, 2077 (1943).
- 17. Clark, E. P., Semimicro quantitative organic analysis, New York (1943).
- 18. Lemieux, R. U., and Purves, C. B., Canad. J. Res., Sect. B, 25, 485 (1947).
- 19. Jorpes, E., and Gardell, S., J. Biol. Chem., 176, 267 (1948).
- 20. Stuart, R. G., Analyst, 72, 235 (1947).

- 21. Wilander, O., Skand. Arch. Physiol., 81, suppl. 15 (1938).
- 22. Wolfrom, M. L., and McNeely, W. H., J. Am. Chem. Soc., 67, 748 (1945).
- 23. Berglund, E., Acta univ. Lundensis, 12, 12 (1875); 13, 4 (1876).
- 24. Berglund, E., J. Chem. Soc., 34, 643 (1878).
- Audrieth, L. F., Sveda, M., Sisler, H. H., and Butler, M. J., Chem. Rev., 26, 49 (1940).

A STUDY OF THE RATE OF OXIDATION OF THE METHYL GROUP OF DIETARY METHIONINE*

By COSMO G. MACKENZIE, JULIAN R. RACHELE, NANCY CROSS, JOSEPH P. CHANDLER, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, October 15, 1949)

We have recently reported that the administration per os to a rat of a single 200 mg. dose of methionine labeled with C¹⁴ in the methyl group was followed by the appearance of one-quarter of the radioactive carbon in the expired carbon dioxide in 24 hours (1). This finding clearly demonstrated the ability of the animal organism to oxidize the biologically labile methyl group to carbon dioxide and water. It also posed the question as to the extent of oxidation of the smaller amounts of methionine methyl that are ingested in one feeding as a part of an average diet. Accordingly, a series of experiments was carried out in an attempt to answer this question.

The amino acid diets employed contained either 0.6 or 1.2 per cent methionine, levels of methionine equivalent to those provided by rations containing 17 and 34 per cent casein. These methionine levels covered the range most frequently encountered in adequate diets of both the purified and natural food types. Both of the diets contained 0.2 per cent choline chloride and 0.4 per cent cystine.

In studying the rate of oxidation of a dietary component in the whole animal with the use of isotopic carbon, it is desirable to determine not only the total amount oxidized during a given period of time, but also the actual changes in the rate of oxidation that occur within the same period, for the sharpness with which the oxidation curve rises and falls reflects the rapidity with which the animal is absorbing the exogenous material, destroying or excreting it, and integrating it with its endogenous counterparts already present in the body. When the experimental animal is given free access to the diet, the shape of the oxidation curve is difficult to interpret, since the amount of isotopic food ingested is not known or controlled for any time interval.

^{*} The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant that has aided greatly in these experiments. A preliminary report on a part of this work was presented before the American Society of Biological Chemists at Atlantic City, New Jersey, March 15, 1948 (Federation Proc., 7, 170 (1948)).

Consequently, in the present study a single 2 gm. portion of the diet containing the radiomethionine was administered by stomach tube to rats which had free access, both before and after this treatment, to the same diet containing ordinary methionine. By determining the amount of radiocarbon dioxide in the expired air collected during hourly intervals following the ingestion of the "radioactive meal," the rise and fall in the rate of oxidation of the methyl group was obtained. The size of the "radioactive meal," approximately one-fifth of the daily food consumption, was dictated by the quantity of radiomethionine required to produce a concentration of radiocarbon dioxide in the expired air that could be measured accurately in the rats receiving the 0.6 per cent methionine diet.

In addition to measuring the per cent of the ingested methyl group converted to carbon dioxide and water, the amount of isotopic methyl carbon excreted in the urine and feces in 24 hours was determined. It was thus possible to compare the 24 hour elimination and retention of exogenous methyl carbon on the high and low methionine diets.

Materials and Methods

The L-methionine labeled with C¹⁴ in the methyl group that was used in these experiments had been previously synthesized in this laboratory by Melville, Rachele, and Keller (2). The barium carbonate prepared from 1 mg. of methionine gave approximately 125,000 c.p.m. when corrected for background and self-absorption.

The metabolism apparatus for the collection of expired CO_2 , urine, and feces and the methods for determining the C^{14} content of these materials have been described in an earlier publication (1). Throughout this paper the C^{14} content of the urine, feces, and expired CO_2 is expressed as a per cent of the ingested methionine, methyl group, or C^{14} . Numerically, all three of these expressions are identical.

The composition of the 0.6 per cent methionine diet is shown in Table I. The 1.2 per cent methionine diet was the same except for the increase in methionine and the corresponding decrease in sucrose. The adequacy of the low methionine diet with respect to labile methyl groups and sulfurcontaining amino acids was shown by the fact that male rats, weighing 130 gm., gained as rapidly in a 21 day period on this diet as on the 1.2 per cent methionine diet. At the end of this time the fat content of their livers was approximately 4 per cent.

In the metabolism experiments, young male rats, obtained from Rockland Farms, New City, New York, were fed one of the experimental diets containing non-radioactive primethionine for a period of 6 to 10 days. When an animal weighed approximately 150 gm., it was given by stomach

tube, at 9.45 in the morning, a single 2 gm. portion of diet in which the ordinary methionine was replaced with L-methionine containing C14 in the methyl group.

The procedure employed in administering the radiomethionine diet was as follows. 400 mg. of the mixed fats (Covo and corn oil, Table I), previously chilled in the refrigerator, were fed to the rat on a small spatula. Next, a stomach tube, consisting of a piece of x-ray catheter with a hypodermic needle inserted in the proximal end, was introduced, and the non-

TABLE I Composition of 0.6 Per Cent Methionine Diet

	per cent
Sucrose	54.8
Covo*	19.0
Corn oil	1.0
Salt mixture†	4.0
Amino acid mixture‡	20.0
Methionine	0.6
L-Cystine	0.4
Choline chloride	0.2
Vitamins§	

- * A hydrogenated vegetable oil.
- † Osborne-Mendel salt mixture, No. 1 (3), Eimer and Amend, New York.
- ‡ The proportion of individual amino acids per 185 parts of mixture is as follows: glycine 1, hydroxy-L-proline 1, L-proline 2, serine 2, L-aspartic acid 2, alanine 4, L-tryptophan 4, L-arginine hydrochloride 6, L-histidine · HCl·H₂O 7, L-tyrosine 10, threonine 14, phenylalanine 15, isoleucine 18, valine 20, L-glutamic acid 20, leucine 26, L-lysine hydrochloride 19, NaHCO₂ 14.
- § The composition of the vitamin mixture in mg. or units per kilo of diet is as follows: thiamine hydrochloride 10, riboflavin 10, pyridoxine hydrochloride 10, nicotinic acid 10, calcium pantothenate 50, inositol 100, p-aminobenzoic acid 10, folic acid 1, biotin 0.1, α-tocopherol acetate 40, 2-methyl-1,4-naphthoquinone 1, vitamin A 7200 units, vitamin D 1200 units.

lipide part of the diet (less methionine) suspended in 1.5 ml. of water was fed through the tube. This was followed immediately by the administration from a 1 ml. syringe of the radiomethionine (12 or 24 mg.) dissolved in 0.5 ml. of water. Finally, 0.5 ml. of water that had been used to wash out the methionine weighing bottle was given through the stomach tube. About 1 minute was required to give the non-lipide portion of the diet plus methionine.

After the stomach tube had been withdrawn, the animal was transferred at once to the metabolism apparatus and the urine, feces, and expired carbon dioxide were collected for the next 30 hours. During this time the

rat had continuous access to the diet containing non-radioactive methionine. No food was eaten during the day. Food consumption during the night was normal when the 2 gm. that had been given by stomach tube were taken into consideration.

RESULTS AND DISCUSSION

In the experiments reported in this paper, two rats were fed the 0.6 per cent methionine diet and four rats the 1.2 per cent methionine diet. In every case the oxidation of the methyl group of the labeled methionine began soon after the ingestion of the diet, as shown by the presence of C¹⁴ in the carbon dioxide collected during the 1st hour. In two of the four animals receiving the high level of methionine the rate of oxidation reached its maximum in the 2nd hour and declined slightly during the 3rd hour. The other rats on this diet, as well as the two animals on the low methionine diet, attained their respective maximum rates of oxidation of the methyl group during the 3rd hour. After the 3rd hour the rate of oxidation in every animal declined sharply. Low and relatively constant rates of oxidation were reached at the 7th hour by the animals on the 0.6 per cent methionine diet, and at the 7th or 8th hour by the rats on the 1.2 per cent diet.

The average curves showing the rate of oxidation of the methyl groups of dietary methionine to carbon dioxide¹ and water by the two sets of rats are given in Fig. 1. While differing considerably in magnitude, they possess the same general shape, and it is the significance of their shape with respect to the metabolism of the ingested methyl group that will be first discussed.

As indicated by the oxidation curves, the metabolism of the methyl group of dietary methionine falls into two phases; the first is characterized by a rapid rise and fall in the rate of oxidation, and the second by the establishment of a relatively steady state. For convenience the descriptive terms "period of assimilation" and "period of equilibrium" may be used to indicate these two phases. In the period of assimilation the rise

¹ The sensitivity with which the oxidation curves of the methyl group mirror the actual rates of conversion of labeled methyl radicals to CO₂ in the body is indicated by the report of Gould and coworkers (4) that the maximum concentration of C¹⁴O₂ in the expired air occurs within 10 minutes after the injection of radioactive bicarbonate. The figures given in the present paper for the oxidation of the methyl group have not been corrected for the C¹⁴O₂ that was formed but subsequently entered into synthetic reactions in the body. According to Gould and coworkers (4) over 90 per cent of an injected dose of bicarbonate is eliminated as respiratory CO₂ in 4 hours. Since in our experiments the urine was collected in acid, any C¹⁴O₂ excreted as bicarbonate was collected along with the respiratory C¹⁴O₂.

in the rate of oxidation is a measure of the rate of absorption of the exogenous methionine and of the concentration of the tagged methyl group at the active centers of oxidation. The almost equally rapid decline in the rate of oxidation after the 3rd hour reflects a fall in the concentration of radiomethyl at the active centers of oxidation. While the process of oxidation will in itself lower the concentration, earlier observations on the chemical and anatomical distribution in the body of the methyl group administered as methionine (1) suggest that concurrent chemical and anatomical

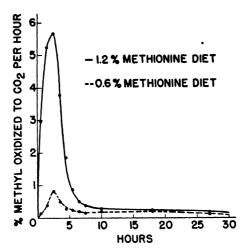


Fig. 1. The rate of oxidation to CO₂ of the methyl group of methionine ingested in a single 2 gm. portion of diet. The diets containing L-methionine labeled with C¹⁴ in the methyl group were administered by stomach tube to 150 gm. male rats which, both before and after this treatment, had continuous access to the corresponding diets containing ordinary methionine. The oxidation curve for the 0.6 per cent methionine diet is an average of two experiments and the curve for the 1.2 per cent methionine diet is an average of four experiments.

translocations of the methyl group also contribute to the decline in the rate of oxidation.

The period of equilibrium was initiated by the establishment of a low and relatively constant rate of oxidation. This indicates that the *major* (most rapid) chemical conversions and anatomical translocations of the

² In vitro studies in this laboratory have shown that the methyl group provided by methionine is oxidized by liver and kidney cortex, but not by heart muscle or testes. There is at present no evidence to indicate whether the initial step in the oxidation can occur while the methyl group is attached to the S of methionine, or whether it takes place only after the methyl group has been transferred to some other molecule.

radiomethyl group have occurred, and that it and its oxidation products² have merged with their counterparts already present in the body. However, as we have previously shown (1), the concentration of the ingested methyl carbon in the tissues and organs is far from uniform even after 52 hours. Presumably a continuous slow redistribution of the radiomethyl

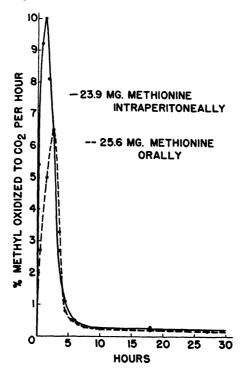


Fig. 2. The effect of oral and intraperitoneal administration on the rate of oxidation to CO₂ of the methyl group of methionine labeled with C₁₄ in the methyl radical. The oral dose was given by stomach tube as a component of 2 gm. of a 1.2 per cent methionine diet. The injected dose was given immediately after the administration of 2 gm. of a methionine-free diet. Before and after treatment both animals (150 gm. male rats) had continuous access to a diet containing 1.2 per cent of ordinary methionine.

group (and its oxidation products) follows this initial rapid but uneven distribution.

⁸ We have isolated radioformaldehyde from liver homogenates incubated with sarcosine labeled with C¹⁴ in the methyl group (5). Moreover, as we reported at the meeting of the American Society of Biological Chemists at Detroit, 1949, the presence of radioactive formic acid has been indicated by the evolution of C¹⁴O₂ when the distillate from the formaldehyde-free homogenate is oxidized with HgCl₂.

In view of the prompt and rapid rise of oxidation during the period of assimilation, the possibility that the intestinal flora contributed significantly to the initial degradation of the exogenous methyl group was investigated. A rat on the 1.2 per cent methionine diet was fed 2 gm. of the ration without methionine and then injected intraperitoneally with 23.9 mg, of radiomethionine. The oxidation curve is shown in Fig. 2. The curve for Rat 3501 (see also Table II), fed 25.6 mg, of radiomethionine in

TABLE II Oxidation and Excretion of Methyl Group of Dietary Methionine

The experimental diet contained 0.4 per cent cystine, 0.2 per cent choline chloride, and 0.6 or 1.2 per cent methionine. Male rats (150 gm.) were fed by stomach tube a single portion of diet in which L-methionine labeled with C14 in the methyl group replaced the ordinary methionine otherwise employed. The oxidation and excretion of the radiomethyl carbon was measured for 24 hours.

	Radioac-	TAMORIONE		Per cent C14 oxidized to C14O2			Per cent C14 excreted*		C14
Rat No.	methionine ingested	0-6 hrs.	6-12 hrs.	12-24 hrs.	Total (A)	Urine (B)	Feces (C)	C14 eliminated (A+B+C)	retained†
	mg.								mg.
3498	11.9	1.5	1.0	2.6	5.1	3.70	0.32	9.12	10.8
3592	12.3	3.2	1.2	2.1	6.5	5.04	0.31	11.85	10.8
3500	24.2	23.4	2.1	3.3	28.8				
3501	25.6	18.8	1.6	2.3	22.7	4.02	0.29	27.01	18.7
3507	24.4	17.6	2.4	3.3	23.3	4.71	0.50	28.51	17.5
3 89	25.3	21.6	2.0	2.8	26.4	4.71	0.34	31.45	17.3
3497‡	23.9	27.1	1.8	3.4	32.3	4.56			

^{*} Not all of the excreted C14 is present as methyl groups; a portion represents their oxidation products.

the diet, is also shown. In so far as can be ascertained by comparing the results of oral and parenteral administration, the intestinal bacteria did not participate extensively in the initial oxidation (or the 24 hour oxidation) of the methyl groups.

The methionine content of the diet had a profound effect on the amount of dietary methyl groups converted to carbon dioxide. On the low methionine diet 6 per cent and on the high methionine diet 25 per cent of the ingested methyl carbon appeared in the expired air as radioactive carbon dioxide in 24 hours. This increase in the proportion of dietary methyl

[†] Expressed as mg. of methionine.

In this rat the radiomethionine was injected intraperitoneally immediately after the administration by stomach tube of 2 gm, of diet from which methionine was omitted.

groups oxidized daily was accomplished almost entirely by a great increase in the rate of oxidation during the period of assimilation, as is shown in Table II. Doubling the methionine content increased the rate of oxidation approximately 9-fold during the first 6 hours. On the other hand, the increase in the rate of oxidation during the period of equilibrium was relatively slight.

The relation between the previous methionine intake and the high rate of oxidation in the period of assimilation on the 1.2 per cent methionine diet was investigated. A rat which had been maintained on the 0.6 per cent methionine regimen was given a 2 gm. portion of the 1.2 per cent radiomethionine diet by stomach tube and then returned to the lower level of methionine. The rate of oxidation (Rat 389, Table II) was just as high in this animal as in those maintained on 1.2 per cent methionine for 7 days before treatment. Consequently, the ability to oxidize the methyl groups at a faster rate on the higher level of methionine was not due to an adaptive increase in the concentration of the oxidative enzymes.

Probably the ingestion of the 24 mg. dose of radiomethionine as contrasted to the 12 mg. dose resulted in the saturation of the synthetic enzymes, thus increasing the proportion of methyl groups available for destruction by the oxidative enzymes. From this point of view, the lower rate of oxidation in rats fed 0.6 per cent methionine was the result of competition for the ingested methyl groups between synthetic and degradative (oxidative) enzyme systems.

An appreciable quantity of the ingested methyl groups was eliminated in the urine in 24 hours in the form of methyl compounds or the oxidation products of methyl groups.⁴ On the 0.6 per cent methionine diet the amount of C¹⁴ present in the urine was nearly as high as the amount of C¹⁴ present in the expired carbon dioxide (Table II). Much smaller amounts were present in the feces. However, doubling the level of methionine had little effect on the per cent of exogenous methyl carbon excreted in either the urine or feces. This is in contrast with the considerable increase in the rate of oxidation during the period of assimilation produced by doubling the methionine intake, and it further emphasizes the contribution of oxidation, in this phase of metabolism, to the regulation of the methyl balance of the body.

Nevertheless, the higher rate of oxidation of the methyl group on the 1.2 per cent methionine diet fell short of reducing the amount of ingested

⁴ One oxidation product is the CO₂ which has been used in the synthesis of urea. The carbon of urea possesses the same specific activity as the carbon of the expired CO₂ (6). Since the amount of urea carbon equals about one-thirty-third of the carbon present in the expired CO₂, the per cent of ingested C¹⁴ present as urea may be approximated by multiplying the per cent present as expired CO₂ by 0.03.

methyl carbon retained by the body in 24 hours to the level that prevailed on 0.6 per cent methionine. The retention of C14 was 10.8 mg. (expressed as methionine) on the low methionine diet and 17.8 mg. on the high methionine diet (Table II).

Since both rations contained an adequate supply of labile methyl groups, and since the animals on both rations gained at the same rate, it does not seem likely that the increased retention of C14 was due to an over-all increase in the storage of methyl groups or their oxidation products. More probably, the ingestion of the high methionine diet increased the oxidation and excretion of non-radioactive methyl groups already present in the body, thus increasing the rate of turnover. Also, raising the methionine level may have increased the oxidation of the methyl groups simultaneously consumed in the form of choline. In either event the retention of newly ingested methyl groups would be increased without appreciably changing the concentration of total methyl groups in the body.

SUMMARY

. The rate of oxidation of the methyl group of dietary methionine has been measured in rats fed diets containing choline and cystine, and either 0.6 or 1.2 per cent methionine. This was accomplished by determining the amount of radiocarbon dioxide expired following the ingestion of a single meal in which C14-labeled methionine replaced the ordinary methionine otherwise present in the diet.

On both diets the oxidation of the radiomethyl group fell into two phases. During the first phase, or period of assimilation, there was a rapid rise in the rate of oxidation to a maximum attained during the 3rd hour, followed by a decline until the 7th or 8th hour. The second phase, or period of equilibrium, was initiated at this time by the establishment of a low and relatively constant rate of oxidation.

Doubling the methionine content of the diet produced a 9-fold increase in the per cent of radiomethyl groups oxidized during the period of assimilation. It had relatively little effect on the rate of oxidation during the period of equilibrium, or on the per cent of radiomethyl carbon excreted in the urine or feces in 24 hours.

The implications of these results are discussed.

The authors wish to acknowledge the technical assistance of Mrs. Marion H. Wilson and Mr. Harry L. Isrow.

BIBLIOGRAPHY

- 1. Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., and du Vigneaud, V., J. Biol. Chem., 180, 99 (1949).
- Melville, D. B., Rachele, J. R., and Keller, E. B., J. Biol. Chem., 169, 419 (1947).

- Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 887 (1937).
- Gould, R. G., Rosenberg, I. M., Sinex, M., and Hastings, A. B., Federation Proc. 7, 156 (1948).
- 5. Mackenzie, C. G., and du Vigneaud, V., Federation Proc., 8, 223 (1949).
- 6. Mackenzie, C. G., and du Vigneaud, V., J. Biol. Chem., 172, 353 (1948).

THE ORIGIN OF THE METHYL GROUP OF EPINEPHRINE*

By ELIZABETH B. KELLER, R. A. BOISSONNAS,† AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

(Received for publication, November 2, 1949)

In the original communication on the effect of choline upon the growth of the rat on a cystine-methionine-free diet supplemented with homocystine (1), it was suggested that, "The methionine-homocystine-choline relationship may be significant, not only to sulfur and fat metabolism, but also to other problems of methylation in the animal organism." Later, when the transfer of methyl groups from methionine to choline and creatine had been demonstrated by the crucial experiment in which deuteriocholine and deuteriocreatine were isolated from the tissues after the feeding of deuteriomethionine to the rat (2), the possibility was raised that the various N-methyl and S-methyl compounds synthesized in the body might derive their methyl groups from the dietary methionine, choline, and betaine.

Our first opportunity to test this hypothesis was afforded by some experiments with the rabbit (3). In addition to choline and creatine, anserine was isolated after deuteriomethionine had been fed. It was found that the methyl group of the isolated anserine was derived from the ingested methionine.

In the present paper this hypothesis has been tested further by an inves-

Ephinephrine

Iodoadrenochrome

tigation of the origin of the methyl group of epinephrine. This work was made possible by the availability of methionine containing radiocarbon

*A preliminary announcement of these results was given in a paper presented before the International Congress of Pure and Applied Chemistry in London in July, 1947.

The authors wish to thank the Lederle Laboratories Division, American Cyanamid Company, for a research grant that has aided greatly in this work.

† Fellow of the Foundation for Scientific Research in the Field of Chemistry (Switzerland).

in the methyl group (4). The radiomethionine was administered to rats and the epinephrine was isolated from the adrenals with the aid of "carrier" epinephrine. It was found that the epinephrine contained a significant amount of radiocarbon. The specific activity of the epinephrine isolated remained constant on recrystallization. The recrystallized ephinephrine was then converted to iodoadrenochrome (5), a quinonoid compound differing markedly from epinephrine in solubility. The specific activity of the iodoadrenochrome was the same as that of the epinephrine within the limits of experimental error.

EXPERIMENTAL

Administration of Methionine Containing C¹⁴ in Methyl Group—Two separate experiments were carried out on two adult rats. The choline-free diet had essentially the same composition as in the experiment of Keller, Rachele, and du Vigneaud (6). The feeding experiments with radioactive methionine were preceded by 3 days feeding of ordinary methionine in order to accustom the rats to the diet. For the duration of the experiment the animals showed the normal gain in weight. The first rat received over a period of 4 days at a level of 1 per cent in the diet 593 mg. of radioactive L-methionine with a radioactivity of 6.49 × 10⁶ c.p.m. per mm. The second rat received over a period of 4 days at a level of 0.6 per cent in the diet 360 mg. of radioactive L-methionine with a radioactivity of 4.22 × 10⁷ c.p.m. per mm. In the case of the second rat, the diet for the pre-experimental period contained 0.6 per cent methionine. The radioactive methionine was prepared by the method of Melville, Rachele, and Keller (4).¹

Isolation of Epinephrine from Adrenals; Recrystallization; Conversion to Iodoadrenochrome—In each experiment the rat was killed with chloroform at the end of the feeding period. The adrenal glands were removed, ground rapidly with sand in a small sintered glass filter, and extracted with six 0.25 ml. portions of cold 5 per cent trichloroacetic acid, each portion being filtered off with gentle suction. To the trichloroacetic acid extract were added 25.0 mg. of dl-epinephrine as a carrier, and a few crystals of sodium sulfite to prevent oxidation of the epinephrine by atmospheric oxygen. The solution was then extracted with five 1.5 ml. portions of peroxide-free ether in order to remove the bulk of the trichloroacetic acid. The solution was filtered and an excess of ammonia was added to the filtrate. The solution was then chilled in an ice bath. The epinephrine which crystallized out was filtered onto a weighed filter paper with an

¹ The BaC¹⁴O₃ used as starting material was supplied by the Monsanto Chemical Company, Clinton Laboratories, on allocation from the United States Atomic Energy Commission.

Allihn type filter (7), washed with alcohol, dried, and weighed. All the samples of epinephrine and iodoadrenochrome were prepared in this way for counting, as a mat of fine crystals having an area of 2.32 sq. cm. and weighing 4 to 9 mg. per sq. cm.

After the radioactivity of the epinephrine was determined by the method described in the next section, it was dissolved off the filter paper with 5 ml. of 0.1 per cent acetic acid. A few crystals of sodium sulfite were added, the solution was filtered, and the epinephrine was recrystallized by adding ammonia. The radioactivity of the recrystallized material was determined.

The recrystallized epinephrine was then converted to iodoadrenochrome by the method of Richter and Blaschko (5). According to these workers and Bergel and Morrison (8), the structure of this compound is indicated to be 2-iodo-3-hydroxy-1-methyl-2,3-dihydroindole-5,6-quinone.

To convert the epinephrine to iodoadrenochrome, it was dissolved in 0.2 ml. of 2 per cent acetic acid, and 0.4 ml. of 4 per cent potassium iodate was added. The dark red crystals of iodoadrenochrome which crystallized out of solution were filtered, washed with water and alcohol, dried, weighed, and analyzed for radiocarbon. In the two experiments 10.3 mg. and 6.7 mg. of iodoadrenochrome were obtained. These amounts are equivalent to 6.2 mg. and 4.0 mg. of epinephrine, respectively.

Radiocarbon Analyses—The radiocarbon analysis on the radiomethionine was carried out by the method described by Keller, Rachele, and du Vigneaud (6). Briefly, this involves the burning of a weighed sample of the radiomethionine, the collection of the CO₂ in alkali, and precipitation of it as BaCO₃ from an aliquot of the alkaline solution. The BaCO₃ is then filtered onto a weighed filter paper, washed, dried, and weighed. The same filtration apparatus (7) was used for the BaCO₃, epinephrine, and iodoadrenochrome samples.

Each sample of epinephrine or iodoadrenochrome was counted, along with the BaCO₃ sample from the radiomethionine, with a thin mica window bell-shaped Geiger-Müller counter and scaling circuit. The activity of each sample was corrected for background and then for self-absorption by using the theoretical self-absorption equation (9), and a value of the absorption coefficient of 0.32 sq. cm. per mg. (10). The specific radioactivities expressed in counts per minute per mm are given in Table I. The over-all error involved in the measurements and calculations was ±8 per cent.

Epinephrine Content of Adrenals—Because of the small amount of epinephrine present in the adrenals of the rat, it was not possible to determine the amount present in the above isolation experiments. However, some preliminary experiments were carried out to determine the approximate

amount of epinephrine in the adrenals of adult rats having about the same weight as those used for the isolation experiments. The epinephrine was extracted from the adrenals by the procedure described above, and the extracts were analyzed for epinephrine by the colorimetric method of Schild (11). This method depends on the oxidation of epinephrine to a red quinone by iodine and has been found to be a reliable colorimetric method for determining epinephrine. The average of two values obtained for the epinephrine in the combined adrenals of an adult rat was 30 γ .

Table I

Radioactivity of Epinephrine Isolated from Adrenals of Rats Fed Methionine

Containing C¹⁴ in Methyl Group

Rat No.	Compound	Weight	Radioactivity
71		mg.	c.p.m. per mu
I (250 gm.)	Radioactive methionine fed	593	6.49×10^{6}
	Epinephrine and carrier, isolated	20.6	9.34×10^{2}
	" " recrystallized	9.3	9.20×10^{2}
	Iodoadrenochrome	10.3	9.77×10^{2}
II (187 gm.)	Radioactive methionine fed	360	4.22×10^{7}
	Epinephrine and carrier, isolated	19.2	4.34×10^{3}
	" " recrystallized	10.7	4.27×10^{3}
	Iodoadrenochrome	6 7	4.47×10^{3}

RESULTS AND DISCUSSION

The constancy of the specific activity of the epinephrine on recrystallization and preparation of a derivative indicates that the radioactivity was incorporated in the epinephrine and not present as an impurity. This point was, of course, quite essential in this experiment, since the amount of epinephrine present in the adrenals is so minute. The derivative selected, iodoadrenochrome, is much less basic than epinephrine, crystallizing out of acetic acid solution. If the activity of the epinephrine had been due to an impurity, it is highly improbable that this radioactive impurity could have been carried down by the iodoadrenochrome crystals in the same molar amount.

In the first experiment in which methionine containing C^{14} in the methyl group was fed to a rat it was found that the methyl group of methionine can be oxidized to carbon dioxide in vivo (12). In the experiment on the first rat described in this paper the radioactivity of the expired carbon dioxide was also measured during the 4 days of feeding the radiomethionine. It was then possible to compare the specific activity of this carbon dioxide with that of the methyl group of the epinephrine on the assumption of an epinephrine content of approximately 30 γ in the adrenals of this rat.

The specific activity of the methyl group of the epinephrine was enormously greater than that of the expired carbon dioxide. From this result we concluded that the radiocarbon in the epinephrine could not have been derived from carbon dioxide and that it must have been introduced into the epinephrine by a transmethylation reaction. We cannot say from these data, however, whether methionine is the immediate precursor of the methyl group of epinephrine or whether an intermediate methyl donor is involved.

The fact that the unmethylated portion of the epinephrine molecule can be derived *in vivo* from phenylalanine has been established by Gurin and Delluva (13), who injected rats with phenylalanine containing C^{14} in the carboxyl group and the α position and isolated epinephrine containing C^{14} from the adrenals by the carrier technique. By a degradation reaction they were able to show that all the C^{14} was in the carbon corresponding to the α -carbon of phenylalanine.

The work of Gurin and Delluva and the present work therefore point to the two essential amino acids phenylalanine and methionine as precursors of epinephrine. The present interest in the interrelationship of arterenol and epinephrine makes the involvement of transmethylation in the biosynthesis of epinephrine all the more intriguing.

SUMMARY

Epinephrine has been isolated by a "washing out" technique from the adrenals of two rats which received methionine containing C¹⁴ in the methyl group. The epinephrine was found to contain a significant amount of C¹⁴. That this C¹⁴ was incorporated in the epinephrine and not present as an impurity was shown by the fact that the specific activity of the epinephrine remained constant on recrystallization and on the subsequent conversion of the epinephrine to iodoadrenochrome.

It has been concluded from these results that the methyl group of epinephrine can be derived from dietary methionine in vivo.

BIBLIOGRAPHY

- du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., Proc. Am. Soc. Biol. Chem., J. Biol. Chem., 128, p. cviii (1939).
- du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., J. Biol. Chem., 140, 625 (1941).
- Schenck, J. R., Simmonds, S., Cohn, M., Stevens, C. M., and du Vigneaud, V., J. Biol. Chem., 149, 355 (1943).
- 4. Melville, D. B., Rachele, J. R., and Keller, E. B., J. Biol. Chem., 169, 419 (1947).
- 5. Richter, D., and Blaschko, H., J. Chem. Soc., 601 (1937).
- 6. Keller, E. B., Rachele, J. R., and du Vigneaud, V., J. Biol. Chem., 177, 733 (1949).
- Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., Ind. and Eng. Chem., Anal. Ed., 18, 349 (1946).

- 8. Bergel, F., and Morrison, A. L., J. Chem. Soc., 48 (1943).
- 9. Libby, W. F., Anal. Chem., 19, 2 (1947).
- 10. Miller, W. W., and Price, T. D., Nucleonics, 1, No. 3, 4 (1947).
- 11. Schild, H., J. Physiol., 79, 455 (1933).
- Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., Melville, D. B., and du Vigneaud, V., J. Biol. Chem., 169, 757 (1947).
- 13. Gurin, S., and Delluva, A. M., J. Biol. Chem., 170, 545 (1947).

THE COMPOSITION OF CERTAIN FRACTIONS OBTAINED BY THE ACTION OF RIBONUCLEASE ON RIBONUCLEIC ACID FROM YEAST

By JAMES E. BACHER AND FRANK WORTHINGTON ALLEN

(From the Division of Biochemistry, University of California Medical School, Berkeley)

(Received for publication, October 1, 1949)

The isolation of a homogeneous preparation of a nucleic acid is yet to be reported. However, sufficient information on purified heterogeneous preparations has accumulated during the last decade to indicate that ultimately a number of structurally different nucleic acids may be found. The largest unit which at present is recognized as a well characterized constituent of the nucleic acids is the mononucleotide. Precise information concerning the order and mode of arrangement of the mononucleotides in the nucleic acids is lacking. Seemingly then to bridge the gap between the mononucleotide and the macromolecular nucleic acids, the isolation and characterization of polynucleotide fragments of nucleic acids become obligate.

At present the only easily controlled fragmentation of ribose nucleic acids is that which is catalyzed by the enzyme ribonuclease. Apart from the work of Loring and Carpenter (1), who have reported that mononucleotides may be isolated from the products of the action of ribonuclease upon a preparation of ribonucleic acid from yeast, it is commonly assumed from the prior work of others (2–5) that the fragments which result from such action are largely polynucleotide in character.

Owing to similarities in their chemical and physical properties the fractionation of mixtures of polynucleotides presents a difficult task. An initial gross separation of the products resulting from ribonuclease action may be brought about by the use of dialysis. The formation of an acid-insoluble, non-dialyzable fraction as a result of the action of ribonuclease upon ribonucleic acid was first noted by Schmidt and Levene (2) and later confirmed by Kunitz (3). Fischer, Böttger, and Lehmann-Echternacht (5) subjected purified, dialyzed ribonucleic acid to the action of ribonuclease, dialyzed the digest, and calculated the average molecular weight for the components present in the dialyzable solute to be 690. Loring, Carpenter, and Roll (6) dialyzed the reaction mixture and recovered the non-dialyzable residue in two fractions. One fraction was obtained by the use of glacial acetic acid as a precipitant, while the other fraction was obtained by the use of alcohol. Tests for solubility or homogeneity of the fractions which were obtained in this manner were not reported.

Certain published (7) and unpublished experiments conducted in this laboratory, in which the foregoing techniques or combinations of techniques were used, gave results which varied according to the time of dialysis and manner of precipitation of the solute from the digestion mixture. Consideration of the varied results led to the conclusion that the dialyzable fractions which result from the action of ribonuclease are not of constant composition and might contain labile linkages susceptible to chemical separation. Thus in order that successive dialysates and ultimately the residue should represent so far as possible the successive products of enzymic action, an experiment was devised in which reactions were carried out in the cold, dialysates were collected after short intervals of time, and the solute was recovered by lyophilization.

EXPERIMENTAL

A commercial sample of ribonucleic acid from yeast (Schwarz) was purified by the procedure described by Levene and Bass (8) with the additional steps of deproteinization by the method of Sevag, Lackman, and Smolens (9). The final product in the form of its sodium salt at pH 7.0 was dialyzed against distilled water and recovered by lyophilization.

15 gm. of this ribose nucleic acid were dissolved in 450 ml. of boiled, distilled water which had been cooled to 10°. The pH was adjusted to 7.0 by the use of a dilute solution of sodium hydroxide. 45 mg. of crystalline ribonuclease (Armour and Company) were added. The solution was placed in dialysis tubing (Visking sausage casing) with a flat width of 13 inches. The tubing was covered with cold, boiled, distilled water and placed in a cold room at approximately 5°. At the intervals given in Table I the dialysate was removed and replaced by cold, boiled, distilled In each case the dialysate was immediately lyophilized. Near water. the conclusion of dialysis the residue had become considerably diluted by The level of the interior solution during the formation of Fraction 5 of the dialysate rose above the level of the exterior solution. In the collection of the remaining fractions the interior solution was kept submerged. Since it was considered that the dilution might prevent complete dialysis of dialyzable material, the experiment was concluded by lyophilizing the residue after collection of Fraction 10 of the dialysate. A 2.0 gm. sample of this residue was dissolved in 50 ml. of water. The pH was adjusted to 7.0 and dialysis was continued for 4 more days. Fraction 11 of the dialyzable material and residue were lyophilized and recovered. A control of 3.0 gm. of the original ribonucleic acid was dialyzed in parallel with the experiment. The total dialysate and residue from the control were recovered by lyophilization.

Total nitrogen was determined by duplicate micro-Kjeldahl analyses.

Composition of Dialysates, Residues, and Controls Oblained by Action of Ribonuclease on Ribonucleic Acid from Yeast TABLE I

Dialysate fraction No.	1	7	<u>س</u>	3 4	2	9	7	•	٥	10	=	Residue	Control	Control
Dialysis time per fraction, krs. Weight of fractions, gm.	2.5	2.5 6 11 25 1.3 1.8 1.6 1.8	11 1.6	1.8	9.5	9.0	23	23 24 0.3 0.2	22.5	22.5 27 0.2 0.4	96 0.8*	1.3*	dialysace 169 0.2	2.5
	13.14	13.28	13.41	14.12	14.43	13.01	13.24	13.32	14.30	14.90	13.1413.2813.4114.1214.4313.0113.2413.3214.3014.9013.70	15.40	11.40	13.90
Guanine N %	8	83	31	33	35	33	<u></u>	35 38 38 40	8	#	35	47	34	35
	8	29	62	20	72	69	0/	7.5	74	62	7.5	83	72	29
Total P, %	8.48	8.69	8.70	8.51	8.35	7.73	7.84	7.74	7.84	8.08	8.48 8.69 8.70 8.51 8.35 7.73 7.84 7.74 7.84 8.08 7.89	8.14	89.9	8.37
Labile P % Total P ' %	41	94	42	42 51 55	22	51 52	52	35	55 60 64		28	69	25	51
	1.55	1.53	1.54	1.66	1.73	1.68	1.69	1.72	1.82	1.84	1.55 1.53 1.54 1.66 1.73 1.68 1.69 1.72 1.82 1.84 1.74	1.89	1.71	1.66
Non-purine N atoms	2.3	2.3	2.2	2.2	2.4	2.4	2.3	2.4	2.6	2.3 2.3 2.2 2.2 2.4 2.4 2.3 2.4 2.6 2.4 2.3	•2.3		2.3	2.5
% soluble in uranyl reagent	. 51 53	53	47	47 34 26	82	33	32	22	=	33 32 27 11 10 27	27		2	

* Residue which remained after Fraction 10 was 3.4 gm. A 2.0 gm. sample of this residue was dialyzed to obtain Fraction 11 (see the text).

Statistical analysis of the data for total nitrogen indicated that 95 per cent of the means of the duplicate analyses were within 0.7 per cent of the true value.

The content of total purine nitrogen was determined by the use of the method of Kerr and Seraidarian (10) which was modified in certain minor details. The amount of copper sulfate which was used was three-quarters of the recommended amount. Hydrolysis was conducted in an N solution of hydrochloric acid for 45 minutes in a boiling water bath. The method was tested on three samples which contained 0.665 mg. of guanine nitrogen in the presence of 2 moles of cytidylic acid per mole of guanine; the average recovery of guanine nitrogen was 102 per cent. The average recovery of pure guanine from solutions in which guanine was the only solute was 97 per cent.

After the isolation of purines in duplicate by the method of Hitchings and Fiske (11), guanine was determined in triplicate by the colorimetric procedure described by Hitchings (12). Analysis of the data indicated that 95 per cent of the means of duplicate isolations are within 3 per cent of the true value. At this point a word of caution regarding the direct application of Hitchings' method to the hydrolysates of nucleic acids or derivatives may be in order. Loring and coworkers (6) have stated that "guanine is the only compound known to be present in nucleic acid that gives a positive reaction." However, under the conditions that are necessary to hydrolyze purines from their derivatives, furfural will form from free ribose. Furfural reacts with the phenol reagent. Comparison experiments performed in this laboratory have yielded high results for guanine when Hitchings' procedure has been applied directly to hydrolysates of nucleic acids and of purine nucleosides.

The partition of phosphate was performed as described by Bacher and Allen (13). Analyses for the content of purines and labile phosphate have been performed on a number of samples. In the most highly purified samples of nucleic acids and in those fractions which were derived by the action of ribonuclease, the labile phosphate corresponded, within 3 per cent, to the content of purines. However, the dialysates from partially purified nucleic acids, certain samples of partially purified nucleic acids, and one highly purified sample which had been in storage for 7 years did not give quantitative correspondence between labile phosphate and content of purines.

DISCUSSION

The entire experiment was conducted at 5°. The temperature coefficient of the enzyme-catalyzed reaction is much greater than the temperature coefficient of dialysis; hence it could be expected that low temperatures

would permit dialysis to parallel the reaction. Thus, successive dialysates should to a certain degree represent the successive products of the enzymic action. The attainment of the objective of the experiment falls short of the ideal, owing to the lack of information concerning many phases of the problem such as (a) the nature of the action of ribonuclease, (b) the structures of the substrates, and (c) the characteristics of membranes toward dialysis of polynucleotides.

In addition to the preparation of ribonucleic acid which is used in the present experiment, two other preparations from two different commercial sources were purified and analyzed. Within the limits of accuracy of the analytical procedures, all of the preparations contain approximately equimolar quantities of the four constituent mononucleotides. Also the control preparation in this experiment, after continued dialysis for over a week and after lyophilization and analysis, contained equimolar quantities of the four constituent mononucleotides. Studies in progress concerning the solubility of these preparations show that the term ribonucleic acid from yeast, at least as applied to these preparations, designates a mixture of at least three components.

According to the analyses of the fractions of the dialysate (Table I), all of the fractions are heterogeneous. Certain trends in the change of composition may be noted. Fractions 1, 2, and 3 are similar in composition. The molar ratio of the purine-containing constituents to the pyrimidine-containing constituents is approximately 2:3. The same molar ratio for the average components of Fractions 4, 5, and 6 is approximately 1:1. The ratio for the remainder of the fractions of the dialysate, though variable, averages 3:2.

The atomic ratio of non-purine nitrogen to stable phosphorus is a measure of the ratio of uracil to cytosine. Thus, the first few dialyzable fractions contain a preponderance of uracil over cytosine. A preliminary experiment gave the same result (7). The later fractions contain approximately equal amounts of the two pyrimidines. Since the non-purine nitrogen is obtained by difference, caution must be exercised in its use. For the data to have significance the substance should have a high content of pyrimidines and must be free from protein. These conditions are met by the dialyzable fractions but not by the non-dialyzable residues.

The first fractions contain a greater proportion of nucleotides of lower molecular weight than do the later fractions. This is shown by the behavior of the fractions toward precipitation with uranyl ion. Zittle's modification (14) of MacFadyen's uranyl reagent (15) was employed. Studies on mononucleotides have shown that they are soluble in the uranyl reagent (14). Nothing is known concerning the solubility of di- and trinucleotides. Polynucleotides of a higher order are considered to be insoluble. There-

fore, the decreasing order of solubility, which is noted as enzymic action and dialysis proceeds, can be taken as a qualitative measure of the size of the polynucleotides in the fractions. If it is assumed that all of the components which are soluble in the presence of uranyl ion consist of mononucleotides, then approximately one-third of the total dialyzable fraction comprises mononucleotides. Also about two-thirds of the material which might conceivably be in the nature of mononucleotides is formed in the first 20 hours of enzymic action.

Prior work (6, 16, 17) indicates that ribonuclease may catalyze the hydrolysis of a phosphate linkage between certain constituent pyrimidine nucleotides and the adjacent nucleotide. If this assumption is applied, then the first fractions of the dialysate represent predominantly the action of ribonuclease on the part of the nucleic acid molecule, in which the spacing of certain pyrimidine nucleotides is more frequent relative to purine nucleotides than is found with the higher molecular weight components which predominate in the later fractions. Since the non-dialyzable residue, which is polynucleotide in character, contains appreciable quantities of pyrimidines, it should be emphasized that not all of the phosphate linkages between pyrimidine nucleotides and adjacent nucleotides are hydrolyzed by ribonuclease. The specificity of ribonuclease probably is such that not only certain specific linkages but also a certain arrangement of groups adjacent to the linkage are necessary for action. The substrate requirements of the peptidases is a well known example of such specificity.

As mentioned earlier, the analysis of highly purified samples of nucleic acids and of those fractions which were isolated as a result of the action of ribonuclease showed a correspondence between the content of purines and labile phosphate. Thus, the action of ribonuclease does not break pyrimidino-ribosidic bonds. If such bonds were broken, the resulting ribose-3-phosphate would have registered as labile, not stable, phosphate (13).

In the present experiment three-fourths of the material is rendered dialyzable by the action of ribonuclease. In experiments on three other samples of ribonucleic acid, to be reported later, the dialyzable fractions were found to be 62, 72, and 80 per cent.

SUMMARY

Ribonucleic acid from yeast was purified and dialyzed, neutralized to pH 7.0, and subjected to the action of ribonuclease at 5°. The dialyzable fractions which resulted from the enzymic action were collected at intervals and immediately recovered by lyophilization. After the completion of enzymic action and of dialysis, the non-dialyzable residue was recovered by lyophilization.

Analyses indicated the original ribonucleic acid to be composed of approximately equimolar quantities of the four constituent mononucleotides. Fractions of the dialysate were found to be heterogeneous. With progressive enzymic action and collection of dialysate the molar ratios of the purine-containing constituents to the pyrimidine-containing constituents passed from 2:3 through 1:1 to 3:2. The corresponding ratio for the non-dialyzable residue was 7:3.

The bearing of these data on the nature of ribonucleic acid and the mode of action of ribonuclease is discussed.

BIBLIOGRAPHY

- 1. Loring, H. S., and Carpenter, F. H., J. Biol. Chem., 150, 381 (1943).
- 2. Schmidt, G., and Levene, P. A., J. Biol. Chem., 126, 423 (1938).
- 3. Kunitz, M., J. Gen. Physiol., 24, 15 (1940).
- 4. Allen, F. W., and Eiler, J. J., J. Biol. Chem., 137, 757 (1941).
- Fischer, F. G., Böttger, I., and Lehmann-Echternacht, H., Z. physiol. Chem., 271, 246 (1941).
- 6. Loring, H. S., Carpenter, F. H., and Roll, P. M., J. Biol. Chem., 169, 601 (1947).
- 7. Bacher, J. E., and Allen, F. W., Federation Proc., 8, 181 (1949).
- Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 301 (1931).
- 9. Sevag, M. G., Lackman, D. B., and Smolens, J., J. Biol. Chem., 124, 425 (1938).
- 10. Kerr, S. E., and Seraidarian, K., J. Biol. Chem., 132, 147 (1940).
- 11. Hitchings, G. H., and Fiske, C. H., J. Biol. Chem., 140, 491 (1941).
- 12. Hitchings, G. H., J. Biol. Chem., 139, 843 (1941).
- 13. Bacher, J. E., and Allen, F. W., J. Biol. Chem., 182, 701 (1950).
- 14. Zittle, C. A., J. Biol. Chem., 163, 119 (1946).
- 15. MacFadyen, D. A., J. Biol. Chem., 107, 297 (1934).
- 16. Bolomey, R. A., and Allen, F. W., J. Biol. Chem., 144, 113 (1942).
- Schmidt, G., Cubiles, R., and Thannhauser, S. J., Cold Spring Harbor Symposia Quant. Biol., 12, 161 (1947).

A COMPARISON OF PENTOSE NUCLEIC ACID FROM PANCREAS WITH RIBONUCLEIC ACID FROM YEAST

By JAMES E. BACHER AND FRANK WORTHINGTON ALLEN
(From the Division of Biochemistry, University of California Medical School,

Berkeley)

(Received for publication, November 1, 1949)

Owing to the relatively high content of guanylic acid (1-5), the pentose nucleic acid from pancreas has always been considered to be distinct from pentose nucleic acids from other sources. It was noted by Eiler and Allen (6) that their preparations of pentose nucleic acid from pancreas behaved similarly to the ribonucleic acid from yeast when subjected to the action of ribonuclease. Later, it was found that the action of ribonuclease on ribonucleic acid from yeast produced an acid-insoluble, non-dialyzable residue which contains a high percentage of guanine (7, 8). The high content of ribonuclease in pancreatic tissue (9-11), the rapid rate of action of ribonuclease, the mild conditions under which the pentose nucleic acid from pancreas is isolated, and the high content of guanine in such preparations suggest that the preparations which have been reported to date may be mixtures of the native nucleic acid and the more insoluble fragments.

Initial advances in the study of the nature of the native pentose nucleic acid from pancreas may be attained from investigations which relate to (a) the isolation of the nucleic acid by mild procedures which block action by ribonuclease, and (b) comparisons of the non-dialyzable residues which remain after the action of ribonuclease on the pentose nucleic acids from pancreas and from other sources.

EXPERIMENTAL

Four preparations of pentose nucleic acid from pancreas were isolated. The first two preparations were obtained by following the procedure outlined by Jorpes (1, 2), with the additional steps of final deproteinization by the method of Sevag, Lackman, and Smolens (12). The first isolation was made from commercial frozen pancreas glands. Analyses according to methods described in previous publications (8, 13) yielded the following results: total nitrogen 15.90 per cent, ratio of guanine nitrogen to total nitrogen 0.57, ratio of purine nitrogen to total nitrogen 0.75, total phosphorus 8.27 per cent, ratio of labile phosphate to total phosphate 0.67, ratio of nitrogen to phosphorus 1.92.

For the second isolation, pancreatic glands from cattle were sliced and

packed in dry ice immediately upon extirpation. The frozen glands were worked up in small batches so that every possible application of speed and low temperature could be made. Both the crude and the finally purified nucleoproteins which were isolated as a part of the procedure gave qualitative tests for ribonuclease activity (9). Analyses gave results similar to those obtained for the first preparation: total nitrogen 16.67 per cent, ratio of guanine nitrogen to total nitrogen 0.54, ratio of purine nitrogen to total nitrogen 0.80, total phosphorus 8.83 per cent, ratio of labile phosphate to total phosphate 0.67, ratio of nitrogen to phosphorus 1.89.

The third isolation was undertaken in the hope that the ribonuclease could either be washed from the preparation or denatured. The procedure cannot properly be said to follow that of any particular author, although any one step may have been a part of a previously published treatment. Frozen pancreas was ground into a cold 7 per cent solution of trichloroacetic acid. The pH was maintained just acid to methyl orange indicator. The suspension was blended with an electric mixer which was surrouned by a pack of dry ice. After filtration by suction with a layer of Super-Cel on the paper, the cake was resuspended into 1 liter of a cold 2 per cent solution of trichloroacetic acid and refiltered. This procedure was repeated twice. The filter cake was then washed five times with 1 liter portions of cold water. Finally the material was suspended and washed with 1 liter portions of the following alcoholic solutions: 15, 35, 35, 50, 70, and 95 per cent. Separations were accomplished with the aid of a Servall "hi-speed" centrifuge. After the separation, the material was dispersed in an etheralcohol mixture (1:3), warmed on a steam bath, and filtered. The filter cake was ground into a chloroform-alcohol mixture (1:1) and refluxed for 30 minutes. After filtration the cake was washed with an ether-alcohol mixture (1:1) and dried in air. Qualitative tests for ribonuclease activity were positive. The nucleic acid which was isolated from the nucleoprotein had a total nitrogen to total phosphorus ratio of 1.94 and a ratio of labile phosphate to total phosphate of 71 per cent. These results are again similar to those for the first two preparations.

The first three preparations definitely carried ribonuclease into the final stage, in which action of the ribonuclease with the pentose nucleic acid could be assumed. Therefore a fourth preparation was undertaken. Pancreas which was obtained in the same manner as described for the second preparation was extracted to remove ribonuclease by the method of Kunitz (14). The filter cake was then extracted twice with a 50 per cent solution of acetone according to the procedure of Dubos and Thompson (9). The air-dried filter cake was then suspended in water, neutralized to pH 6.0, and the extraction of nucleoprotein undertaken by the technique of Jorpes. However, the extractions and alcoholic precipitation procedures yielded

a nucleic acid slightly contaminated with protein rather than a nucleoprotein. Removal of the contaminating proteins by the method of Sevag, Lackman, and Smolens yielded the sodium salt of a nucleic acid with the following composition: total nitrogen 13.9 per cent, ratio of guanine nitrogen to total nitrogen 0.43, ratio of purine nitrogen to total nitrogen 0.67, total phosphorus 8.1 per cent, ratio of labile phosphate to total phosphate 48.5 per cent, ratio of total nitrogen to total phosphorus 1.72.

The non-dialyzable residues which remained after ribonuclease action on ribonucleic acid from yeast, as well as the non-dialyzable residues which remained after the action of ribonuclease on certain of the foregoing preparations of pentose nucleic acid from pancreas, were prepared by the

Table I

Composition of Non-Dialyzable Residues (Sodium Salts) Obtained by Action of Ribonuclease on Ribonucleic Acid from Yeast and Pentose

Nucleic Acid from Pancreas

	Ribonuo	cleic acid	Pentose nucleic acid		
Total N, %	15.45	15.40	15.45	15.30	
Guanine N Total N, %	48	47	58	51	
Purine N Total N, %	81	82	78	82	
Total P, %	8.08	8.14	8.20	7.88	
Labile P 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7	70	69	68	70	
Total N Total P	1.92	1.89	1.88	1.94	

methods described in previous publications (8, 13). The results of the analyses are presented in Table I.

DISCUSSION

The compositions of the first three preparations of pentose nucleic acid from pancreas are similar to those previously reported in the literature in that the contents of purines and particularly of guanine are relatively high. Since it was demonstrated that ribonuclease was contained in the pancreatic nucleoprotein, which is the intermediate in the isolation of pentose nucleic acid from pancreas by classical procedures, it can be inferred that part of the non-dialyzable fragments are the result of action by trace amounts of ribonuclease upon the original nucleic acid. Thus, it is believed that all samples of pentose nucleic acid from pancreas, which have been isolated to date, represent degradation products of the native nucleic acid.

The fourth preparation of pancreatic nucleic acid is, to our knowledge, the first reported isolation attempted after the removal of ribonuclease. The method chosen for the extraction of ribonuclease, while considered a standard procedure for the isolation of ribonuclease, may bring about certain changes, such as hydrolysis of certain labile bonds, in the nucleic acid. This can only be determined by further study. However, with the exception of the slightly higher quantity of guanine, which is the lowest yet reported for such a preparation, the analyses are remarkably similar to those reported for certain preparations of ribonucleic acid from yeast.

The compositions of the non-dialyzable residues which were produced from both ribonucleic acid from yeast and the first two preparations of pentose nucleic acid from pancreas, after complete action by ribonuclease followed by exhaustive dialysis, are similar. Both types of residues are characterized by increased content of purines and particularly of guanine, in comparison to the composition of the original preparations of the nu-The manner of formation of the four residues, in addition to the similar compositions, suggests that the residues have structural similarities. These structural similarities must therefore exist in the native nucleic acids. Present information does not permit speculation regarding the fragments which are the result of ribonuclease action and which are removed by dialysis. They could be quite different for the two types of nucleic acids. Schmidt, Cubiles, and Thannhauser (15) have recently emphasized certain differences between the behavior of the two types of nucleic acids toward ribonuclease. Their findings can be construed according to the foregoing arguments or that the pancreas does contain a pentose nucleic acid which is resistant to the enzyme.

SUMMARY

It has been demonstrated that ribonuclease is contained in the pancreatic nucleoprotein which is the intermediate in the isolation of pentose nucleic acid from pancreas by classical procedure.

It is believed that all samples of pentose nucleic acid from pancreas reported to date represent degradation products of the native nucleic acid.

The analytical data are given for a preparation of pancreatic nucleic acid that has been isolated after removal of ribonuclease from the pancreas.

The non-dialyzable residues which are obtained by the action of ribonuclease on the pentose nucleic acid from pancreas are compared with those which are obtained in analogous experiments in which ribonucleic acid from yeast is the substrate.

BIBLIOGRAPHY

- 1. Jorpes, E., Acta med. Scand., 68, 503 (1928).
- 2. Levene, P. A., and Jorpes, E., J. Biol. Chem., 86, 389 (1930).

- 3. Jorpes, E., Biochem. J., 28, 2097, 2102 (1934).
- 4. Steudel, H., Z. physiol. Chem., 241, 84 (1936).
- 5. Vischer, E., and Chargaff, E., J. Biol. Chem., 176, 715 (1948).
- 6. Eiler, J. J., and Allen, F. W., Proc. Soc. Exp. Biol. and Med., 46, 436 (1941).
- 7. Loring, H. S., Carpenter, F. H., and Roll, P. M., J. Biol. Chem., 169, 601 (1947).
- Bacher, J. E., and Allen, F. W., Federation Proc., 8, 181 (1949); J. Biol. Chem., 182, 701 (1950).
- 9. Dubos, R. J., and Thompson, R. H. S., J. Biol. Chem., 124, 501 (1938).
- 10. Bain, J. A., and Rusch, H. P., J. Biol. Chem., 153, 659 (1944).
- 11. Greenstein, J. P., J. Nat. Cancer Inst., 4, 275 (1944).
- 12. Sevag, M. G., Lackman, D. B., and Smolens, J., J. Biol. Chem., 124, 425 (1938).
- 13. Bacher, J. E., and Allen, F. W., J. Biol. Chem., 183, 633 (1950).
- 14. Kunitz, M., Science, 90, 112 (1939).
- 15. Schmidt, G., Cubiles, R., and Thannhauser, S. J., Abstracts, American Chemical Society, 115th meeting, San Francisco, 28C (1949).

SOME PROPERTIES OF A FACTOR IN ALFALFA MEAL CAUSING DEPRESSION OF GROWTH IN CHICKS

By D. W. PETERSON

(From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley)

(Received for publication, November 10, 1949)

It has recently been demonstrated that dehydrated alfalfa meal in the diet of young chicks causes a depression in growth proportional to the level of alfalfa meal in the ration (1, 2). The growth depression is accompanied by a decline in food consumption. Most of the inhibitory effect on growth can be removed by exhaustive extraction of the alfalfa meal with hot water. The residue so produced has little growth-depressing action, whereas the water extract produces marked inhibition of growth (2).

The present report describes a crude fractionation of this water extract and some of its properties.

EXPERIMENTAL

Fractionation—The general procedure employed in the fractionation may be broadly outlined as follows: Dehydrated alfalfa meal was extracted overnight with a large volume of distilled water at room temperature. Toluene and chloroform were added to inhibit bacterial growth. The mixture was filtered and the residue extracted five times with a large volume of distilled water at 95°, the extract being pressed out each time in a filter press. The residue was dried at 80°. The combined filtrates were concentrated in vacuo and 95 per cent ethyl alcohol (sp. gr. 0.815) was added until a concentration of 80 per cent alcohol was reached. The resulting precipitate was allowed to settle for several hours and filtered; it was then suspended in boiling water, alcohol was once more added to a concentration of 80 per cent, and the mixture was again filtered. After the precipitate was washed with 80 per cent and 95 per cent alcohol, it was dried at 65°.

The combined alcohol filtrates were concentrated in vacuo to a volume of 1 liter per 4 kilos of alfalfa meal, and to this solution were added 2.66 volumes of 95 per cent ethanol. Acetone was then added with constant stirring. Upon first addition of acetone a dark brown syrupy mass separated out. Further addition of acetone precipitated a light brownish yellow substance which agglomerated into a gummy mass. Fractional reprecipitation of this mixture of acetone-insoluble precipitates yielded two fractions, Nos. I and II. Fraction I was a dark brown hygroscopic

syrup which was extremely difficult to dry. Fraction II was obtained as a gummy yellowish mass which dried to a light brown solid. The supernatant liquids from the numerous reprecipitations of the acetone-insoluble material were decanted through filter paper and combined. After standing for several days, a white crystalline substance (Fraction III) precipitated from this solution.

When possible, the experimental fractions were dried and finely ground. Those that were difficult to dry were dissolved in 50 per cent ethanol and dried on the barley used in the experimental diet.

Growth Tests-The biological tests employed to detect the presence or absence of the growth-inhibiting factor were growth tests on 7 to 14 dayold single comb white Leghorn chicks that had been previously maintained on a standard stock diet. The chicks were selected for uniformity of weight and rate of growth and were maintained on the experimental diets for periods varying from 2 to 3 weeks. The basal diet, which was formulated to fulfil all the known nutritive requirements of the chick, consisted of the following ingredients: ground barley, 30 per cent; ground corn, 22 per cent; ground wheat, 10 per cent; wheat bran, 10 per cent; fishmeal, 7.5 per cent; soy bean oil meal, 10 per cent; dried whey, 2.5 per cent; dried skim milk, 2.5 per cent; liver meal, 2 per cent; ground limestone, 1.5 per cent; special steamed bone meal, 1.2 per cent; sodium chloride, 0.5 per cent; MnSO₄·H₂O, 0.025 per cent; fortified fish oil (containing 3000 units of vitamin A and 400 of vitamin D per gm.), 0.25 per cent; and riboflavin, 2 mg. per kilo. The stock diet was used as a control diet. It contained the same ingredients as the basal diet, except for the barley, which was replaced by the following constituents: corn, 8 per cent; wheat bran, 4.5 per cent; alfalfa meal, 4 per cent; barley, 10 per cent; and wheat, 3.5 per cent. The basal diet and the stock diet produced parallel growth results in all trials. The experimental diets contained either 20 per cent alfalfa meal or the prepared fractions. These were substituted for a like quantity of barley in the basal diet. The 20 per cent level of alfalfa meal had been found consistently to produce growth depression. The various fractions prepared from alfalfa meal were usually fed at levels somewhat above that which would be equivalent, on the basis of solids, to 20 per cent alfalfa meal, in order to allow for losses in preparation or for incomplete extraction.

The results recorded in Table I show that the growth depressant may be extracted by water and that repeated extraction with hot water is more efficient than extraction at room temperature.

Neither the precipitate obtained with 50 per cent alcohol nor that obtained with 80 per cent alcohol had growth-depressing action (Table II). The precipitate obtained by addition of acetone to an aqueous alcohol solu-

TABLE I

Growth-Depressing Effects of Aqueous Extracts of Alfalfa Meal
8 day old chicks, eight chicks per group.

Diet	Equivalent of alfalfa	Average gain in weight, 21 days
	per cent	gm
20% alfalfa	20	85
15% residue cold water extraction	21	129
13% "hot "extraction	20	151
13% " cold " and hot water extraction	21	156
7% cold water extract	34	79
7% hot " "	29	62
5 25% hot water extract of cold water extracted residue	70	52
Stock diet	4	169
Basal " (no alfalfa)	0	184

Table II

Effect on Growth of Chicks of Various Fractions Obtained from Aqueous Extract of
Alfalfa Meal

Eight chicks per group

		Equiva-	Average gain in weight		
	Diet	lent of alfalfa 14 days		21 days	
		per cent	gm	gm	
Trial I, 9 day-old chicks	20% alfalfa	20	38		
	2 4% 50% ethanol ppt	70	81		
	7% filtrate solids	37	35		
	Basal diet (no alfalfa)	0	81		
Trial II, 7 day-old chicks	20% alfalfa	20	32	57	
	3% 50% ethanol ppt	83	92		
	2 8% 80% ethanol ppt	89	89		
	5% ethanol-acetone ppt.	90	21	37	
				(4 dead, 7 dead by 36th	
		1 1		day)	
	5% ethanol-acetone fil- trate	55	69	130	
	Stock diet	4	85	149	
	Basal "	0	88	157	

tion had marked growth-depressing activity, but the filtrate had almost none (Table II). Of eight birds on the diet containing the alcohol-acetone precipitate, four were dead within 3 weeks, and seven were dead by the 36th day. Those birds examined immediately after death were extremely emaciated, showing atrophy of the leg and breast muscles, ascites and watery blood; the general appearance resembled starvation, although the alimentary tract in some cases was found to contain food.

During the course of the fractionation it was noted that the aqueous solutions of the fractions later found to have growth-depressing properties foamed strongly. This characteristic suggested the presence of saponins, and thus a possible explanation of the toxicity of alfalfa meal to chicks. It was first shown by Ransom (3, 4) that saponins react with sterols to form addition compounds which no longer possess toxic properties and which have lost the ability to hemolyze red blood cells. To test the possibility that a sterol might counteract the growth-depressing agent, cholesterol was added to an alfalfa-containing diet.

The results of this test indicated that cholesterol largely counteracted the growth-depressing action of alfalfa meal (Table III). In further ex-

Table III

Effect of Cholesterol on Toxicity of Alfalfa Meal

week-old chicks, twelve chicks per group.

Diet		Average gain in weight		
		21 days		
	gm	gm.		
20% alfalfa .	56	117		
20% " + 0 5% cholesterol	90	161		
20% " + 1% cholesterol	102	176		
Basal diet (no alfalfa)	117	197		

periments, which need not be described here, cholesterol was found repeatedly to have a definite effect in counteracting the growth depressant.

An experiment was then carried out to determine the relative potency of the inhibitory fractions (Table IV). Fractions I and II and the alfalfa meal were fed both with and without the addition of cholesterol. The acetone precipitate and the fractions obtained from it (Fractions I and II) were found to be more active as growth depressants than the original aqueous extract, indicating considerable concentration of the growth inhibitor. The toxicity of both Fractions I and II was to a large degree counteracted by cholesterol. The group fed Fraction I was maintained only 12 days because of lack of material. Fraction III was not tested in this trial since it had been found in a preliminary experiment to have no growth-depressing action. In one group (Table IV) a phytosterol (Eastman), of unknown origin, appeared also to have some effect in counteracting the growth inhibition.

Hemolytic Tests—If saponins were responsible for the toxicity of alfalfa meal, the growth-depressing fractions should possess hemolytic properties

Table IV

Growth-Depressing Effects of Various Fractions from Alfalfa Meal
9 day-old chicks, eight chicks per group.

Diet	of alfalfa -	Average gain in weigh	
Diet	of alfalfa	12 days	21 days
	per coni	£m.	£m.
20% alfalfa	20	28	72
20% " + 1% cholesterol	.20	61	137
3% aqueous extract	10 7	67	153
3% ethanol acetone ppt	31	45	94
3% Fraction I	133	36	
3% " " + 1% cholesterol	133	82	
3% " II	41	40	85
3% " " + 1% cholesterol	41	71	154
Basal diet (no alfalfa)	0	78	168
Stock "	4	79	174
20% alfalfa + 1% phytosterol (Eastman)	20	52	126

Table V

Comparison of Growth Depression and Hemolysis by Various Fractions
from Alfalfa Extract

Sample	Growth depression*	Hemoly- sus†
Hot water extract	+	+
" " + cholesterol	Not tested	_
80% alcohol ppt.	-	
Alcohol-acetone ppt	+	+
" + cholesterol	Not tested	-
Fraction I	+	+
" + cholesterol	_	-
" II	+	+
" + cholesterol	-	-
" III	l –	_

^{* +} indicates growth depression, - little or no growth depression.

which should be counteracted by treatment with cholesterol. All fractions were made up in duplicate as 1 per cent solutions in 0.9 per cent sodium chloride. Digitonin, a saponin of known hemolytic activity, was used as a control, in 0.1 per cent solutions. To one of each pair of duplicate solu-

^{† +} indicates hemolysis, - no hemolysis.

tions of alfalfa fractions and of digitonin was added enough cholesterol to make a 0.5 per cent suspension. All solutions were then heated for $\frac{1}{2}$ hour in a boiling water bath, allowed to stand for several hours, and filtered. The hemolytic properties of each solution were then determined, by the use of a 2 per cent suspension of washed rat erythrocytes in isotonic saline solution as in the procedure described by Kofler (5).

All samples having growth-depressing action also had hemolytic action. The cholesterol treatment prevented hemolysis in all instances (Table V). Samples that did not depress growth did not hemolyze erythrocytes. All samples except Fraction I caused complete hemolysis at a dilution of 1:800; Fraction I caused complete hemolysis at 1:400. Similar results were obtained by substituting one sample of chicken erythrocytes for rat erythrocytes. The hemolytic index was not determined, since none of the samples was considered sufficiently pure for this test.

DISCUSSION

Fractions obtained from an aqueous extract of alfalfa meal produced depression of growth in chicks. The strong foaming properties of these fractions suggested saponins as the growth-depressing agent. Experimental evidence for this possibility was obtained in the finding that growth-depressing fractions of alfalfa hemolyzed red cells and that treatment with cholesterol inactivated the hemolytic properties. Further evidence was found in feeding tests, which indicated that the growth-depressing activity of alfalfa could be largely counteracted by the inclusion of cholesterol in alfalfa-containing rations.

Reports in the literature concerning the presence of saponins in alfalfa are extremely few. Jacobson (6) in 1919 prepared from alfalfa a substance which foamed strongly in aqueous solutions and which he described as a saponin. Since this material contained nitrogen and since it failed to hemolyze red blood cells, some authors have doubted that it should be considered a saponin (5, 7). Jaretzky, however, using the hemolytic test, found evidence for the occurrence of saponins in twenty species of *Medicago*, including *Medicago* sativa (alfalfa) (7, 8). It is therefore possible that Jacobson's "alfalfa saponin" was a crude preparation and might have undergone some inactivation owing to the method of preparation.

Until saponins are isolated from alfalfa meal in pure form and are shown by feeding tests to cause growth depression, it cannot be stated categorically that they are responsible for the growth-inhibiting properties of alfalfa meal. The present report, however, strongly indicates that any further investigations of the deleterious component, or components, of alfalfa must consider the possibility that saponins may account wholly or in part for its growth-inhibiting effects.

SUMMARY

- 1. Concentrates which depressed the growth of chicks were obtained by fractionating a hot water extract of alfalfa meal. The active principle was soluble in 50 and 80 per cent ethanol and was largely precipitated from an aqueous alcohol solution by the addition of acetone.
- 2. The growth-depressing action of this material was to a great extent counteracted by simultaneous feeding with cholesterol.
- 3. The inhibitory concentrates had hemolytic properties which were destroyed by boiling the concentrates with cholesterol. The hemolytic properties, together with the observed foaming action, strongly suggest that the material involved is a saponin.

BIBLIOGRAPHY

- 1. Cooney, W. T., Butts, J. S., and Bacon, L. E., Poultry Sc., 27, 828 (1948).
- 2. Lepkovsky, S., Shaeleff, W., Peterson, D., and Perry, R., Poultry Sc., in press.
- 3. Ransom, F., Deut. med. Wochschr., 27, 194 (1901).
- 4. Ransom, F., Biochem. J., 16, 668 (1922).
- 5. Kofler, L., Die Saponine, Vienna (1927).
- 6. Jacobson, C. A., J. Am. Chem. Soc., 41, 640 (1919).
- 7. Jaretzky, R., Angew. Botan., 22, 147 (1940).
- 8. Jaretzky, R., and Lindner, W., Arch. Pharm., 277, 45 (1939).

THE BIOLOGICAL ACTIVITY OF α -TOCOPHERYLHYDROQUINONE AND α -TOCOPHERYLQUINONE*

By JULIA B. MACKENZIE, HARRIS ROSENKRANTZ, STANLEY ULICK, AND ADE T. MILHORAT

(From the Departments of Psychiatry and Medicine, Cornell University Medical College, the Russell Sage Institute of Pathology, and The New York

Hospital, New York)

(Received for publication, November 2, 1949)

A major obstacle in relating the biochemical action of α -tocopherol with an oxidation-reduction system in the body has been the large and apparently convincing volume of evidence indicating that its p-quinone, α -tocopherylquinone, and α -tocopherylhydroquinone are devoid of vitamin E activity.

Thus Karrer and coworkers (2), John, Dietzel, and Emte (3), Wright and Drummond (4), Karrer and Geiger (5), and Golumbic and Mattill (6) found that the quinone was inactive in the rat. Golumbic and Mattill also reported that the hydroquinone, α -tocopherylhydroquinone, was inactive in this species. These same workers (7) found no activity when α -tocopherylquinone was administered orally or parenterally to rabbits deficient in vitamin Ξ . While Evans et al. (8) reported the p-quinone to be almost as effective as α -tocopherol itself in the rat, a 4 mg. dose producing fertility in half of the animals tested, later experiments by Tishler and Evans (9) showed that 100 mg. doses of a well characterized preparation were devoid of activity.

Utilizing the methods employed by Mackenzie and McCollum (10) in their demonstration of the cure of nutritional, muscular dystrophy in rabbits by α -tocopherol, we have found that both α -tocopherylquinone and the hydroquinone are potent sources of vitamin E when injected intravenously. Furthermore, both of these compounds have been shown to cure effectively muscular dystrophy when administered in higher doses per os.

Materials and Methods

 α -Tocopherylquinone was prepared by oxidizing 2 gm. of dl- α -tocopherol¹ with 10 gm. of ferric chloride in 200 ml. of absolute ethanol for 24 hours at

*The authors wish to express their appreciation to the Armour Fund for Research in Muscular Disease and to the Nutrition Foundation, Inc., for funds that greatly aided in these experiments. A preliminary report on a part of this work was presented before a Conference on Vitamin E, New York, April 16, 1949 (1).

¹We are indebted to Merck and Company, Rahway, New Jersey, for generous supplies of dl- α -tocopherol.

room temperature in the dark. At the end of this time the reaction mixture was diluted with water and extracted with ethyl ether. The ether was removed in vacuo under nitrogen and the residue was dissolved in petroleum ether. The quinone was converted to the hydroquinone by hydrogenation with 5 per cent palladium on calcium carbonate as the catalyst. The catalyst was removed by centrifugation and the petroleum ether solution was then chilled at 0° , according to the procedure of Tishler and Wendler (11), whereupon the hydroquinone separated as a waxy solid. The mixture was centrifuged and the solvent was decanted. The α -tocopherylhydroquinone was washed several times with petroleum ether to remove any α -tocopherol or quinone present as a contaminant.

The hydroquinone was dissolved in ethyl ether and reconverted to the quinone by oxidation with silver oxide. The quinone so obtained was reduced and reoxidized by the procedures outlined above until its extinction coefficient did not increase. The extinction coefficients of the two preparations of quinone used in the biological tests were 450 and 455 at 270 m μ . The per cent of reducing substances present in these preparations, expressed as α -tocopherol, was 0.06 and 0.2, respectively, as measured by the method of Emmerie and Engel (12). The hydroquinone prepared by reducing these quinone preparations showed selective absorption at 288 m μ with an extinction coefficient of 88. The purity of both the quinone and hydroquinone was confirmed by infra-red absorption.²

The α -tocopherylhydroquinone employed in the animal tests was prepared immediately before its administration by hydrogenation of the purified quinone suspended in propylene glycol. The catalyst was removed from the hydroquinone, which is soluble in propylene glycol, by centrifugation.

The experimental animals were young male and female chinchilla rabbits weighing from 500 to 700 gm. Muscular dystrophy was produced by maintaining the animals on Diet 13 of Goettsch and Pappenheimer (13). Supplements were administered when the rabbits had developed Stage II dystrophy as defined by Mackenzie and McCollum (10). This stage of dystrophy is characterized by loss of weight, decreased food consumption, a creatine excretion of approximately 80 mg. daily, and moderate paralysis. Food was removed for 5 hours before and 5 hours after the administration of oral supplements to minimize mixing of the test substance and the experimental diet in the stomach. Injected animals were allowed continuous access to the diet. Oral doses were given by stomach tube and injected doses were given in the marginal ear veins.

For purposes of supplementation, the hydroquinone was dissolved in

² Rosenkrantz, H., unpublished data.

propylene glycol, or in propylene glycol containing 10 per cent ethanol.³ A concentration of 20 mg. per ml. was generally employed. When amounts of the hydroquinone larger than 20 mg. were administered, the concentration was increased so that the volume did not exceed 1 ml. Immediately after the administration of the test dose, the hydroquinone content of each preparation employed was determined in an aliquot by a modification of the method of Emmerie and Engel. α -Tocopherylquinone and α -tocopherol were suspended in a minimal quantity of the propylene glycolethanol mixture (less than 0.5 ml.) immediately before administration.

RESULTS AND DISCUSSION

The results of our first experiments on the intravenous administration of single doses of α -tocopherylhydroquinone and α -tocopherylquinone to rabbits with Stage II muscular dystrophy revealed that both of these compounds possess vitamin E activity. The creatine excretion was depressed, muscular weakness disappeared, growth was resumed, and appetite returned to normal. The prompt drop in creatine excretion and the rate of fall are illustrated in Fig. 1.

In view of these positive results, the vitamin E activity of α -tocopherylhydroquinone, α -tocopherylquinone, and α -tocopherol was compared by the assay procedure described earlier by Mackenzie (14). This method is based on the correlation between the amount of α -tocopherol administered in a single dose and the duration of the fall in urinary creatine. The results of our studies on the comparative activity of these compounds administered intravenously are summarized in Table I. At a 5 mg. level the activity of the hydroquinone was approximately equal to that of α -tocopherol. However, increasing the amount of hydroquinone injected over a range of 5 to 25 mg. did not prolong the period of depressed creatinuria, indicating that excess amounts were not stored but were either excreted or destroyed. With α -tocopherol, on the other hand, increasing the dose prolonged the response. This finding demonstrated that the activity of the hydroquinone was not due to the presence of α -tocopherol as a contaminant.

Intravenous α -tocopherylquinone was less active than the hydroquinone (Table I). At the 5 mg level the quinone had no effect on the creatinuria. While a definite response was produced by 10 mg., it was not until the dose was raised to 50 mg. that the duration of the effect was comparable to that obtained with 5 mg. of the hydroquinone. It should be noted, however,

³ A suspension of the hydroquinone in 0.9 per cent NaCl, while not satisfactory for routine use because of its physical properties, produced the same physiological response. Propylene glycol itself has no curative action in dystrophic rabbits.

that the injected hydroquinone was in solution, whereas the quinone was suspended in the menstruum.

Vitamin E activity was also observed when single doses of the hydroquinone and the quinone were administered by stomach tube. As shown in Table II, the hydroquinone was the more active of the two compounds. In both cases, however, there was greater activity when the compounds were injected intravenously than orally. Presumably, the reduction in activity of both substances was due to poor absorption, to partial destruc-

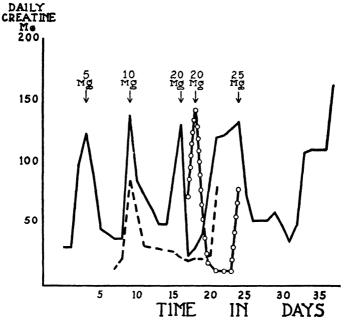


Fig. 1. Depression of creatinuria in dystrophic rabbits following the intravenous administration of single doses of α -tocopherylhydroquinone, α -tocopherylquinone, and α -tocopherol. Solid curve, Rabbit 17, α -tocopherylhydroquinone; broken line, Rabbit 27, α -tocopherol; O, Rabbit 90, α -tocopherylquinone.

tion in the gastrointestinal tract, or to inactivation in the body before they reached the systemic circulation. In any event, the lower potency of the quinone and hydroquinone when administered *per os*, and the inability of the body to store them efficiently, may explain the failure of other workers to detect their vitamin E activity.

Having observed that both the quinone and hydroquinone, when given in a single dose, elicited a response in the dystrophic rabbit qualitatively indistinguishable from that produced by α -tocopherol, we carried out ex-

Table I

Effect of Intravenous Administration of α-Tocopherylhydroquinone, α-Tocopherylquinone, and α-Tocopherol on Creatine Excretion of Dystrophic Rabbits

No. of	Supplement	Dose	Average daily creatine excretion at time of	Average maximal drop in creatine	Duration of lowered creatine excretion	
			supplement	excretion	Average	Extremes
		mg.	mg.	per cent*	days	days
4	α-Tocopheryl- hydroquinone	5	120	71	5.5	4-6
2		10	115	63	7.3	6-7
1		15	102	71	8.0	
4		20	150	• 84	5.2	4-6
2		25	83	72	6.5	5-8
1		50	104	85	8.0	
2	α-Tocopherylquinone	5	113	0	0	
2		10	98	77	2.0	
6		20	120	85	3.0	2-5
4		50	117	72	4.8	4-6
6,		100	145	89	12.6	8-16
2	α-Tocopherol	5	110	82	6.5	5-8
1		10	87	75	12.0]
2		20	112	82	14.0	13-15
1		50	113	93	26.0†	

^{*}Maximal drop in creatine (mg.)
Creatine (mg.) at time of supplement × 100

Table II

Effect of Oral Administration of α -Tocopherylhydroquinone, α -Tocopherylquinone, and α -Tocopherol on Creatine Excretion of Dystrophic Rabbits

No. of	Supplement	Dose		Average maximal drop in creatine	Duration of lowered creatine excretion	
			time of supplement	excretion	Average	Extremes
		mg.	mg.	per cent*	days	days
1	α -Tocopherylhydro- quinone	5	110	0	0	0
2		20	121	70	3.5	3-4
2		100	98	75	7.5	7-8
4 `	α-Tocopherylquinone	50	156	0	0	
2		100	122	73	4.5	4-5
2		200	104	70	7.5	7-8
3	α -Tocopherol	20	104	76	15.6	11-20

^{*}Maximal drop in creatine (mg.)
Creatine (mg.) at time of supplement × 100.

[†] Animal sacrificed; creatine excretion 15 mg. at time of death.

periments to determine whether or not they could replace α -tocopherol for longer periods of time. Three rabbits with Stage II dystrophy and with a creatine output of 130, 152, and 162 mg., respectively, were injected daily with 12 mg. of α -tocopherylhydroquinone. In each of the animals the creatine excretion fell to within the normal range of 10 to 15 mg. per kilo of body weight within 2 days, and there was a prompt remission of physical symptoms. Two of the animals were sacrificed after 10 days and the third one at the end of 7 weeks. Histological sections were prepared from the thigh muscles of each rabbit and examined for the presence of the lesions of muscular dystrophy described by Goettsch and Pappenheimer (13). None were found.

Three control rabbits with creatine excretions of approximately 100 mg. per day had extensive muscle lesions. Moreover, Mackenzie, Levine, and McCollum (15) have shown that on the experimental diet employed muscle lesions invariably are present even in those rabbits exhibiting a mild creatinuria. It may be assumed, therefore, that lesions were present in our animals when therapy was initiated and that these lesions were cured and their recurrence prevented by the hydroquinone.

The effectiveness of the quinone in substituting for α -tocopherol as a source of vitamin E over prolonged periods was demonstrated by curing animals of repeated attacks of dystrophy with single doses of this compound administered either intravenously or orally. The injected doses ranged from 20 to 100 mg.; the oral doses were either 100 or 200 mg. Five rabbits were cured of from three to seven attacks of Stage II dystrophy during periods that ranged from 11 to 19 weeks. Had they been given no supplement, these animals would have succumbed to their first attack of the disease within a week or 10 days (10).

With the demonstration of the biological activity of α -tocopheryl-quinone and α -tocopherylhydroquinone, a major obstacle in relating the biochemical action of vitamin E with an oxidation-reduction system has been removed, and a reconsideration of its reactions in the body is necessary.

Michaelis and Wollman (16) have recently observed the reversible formation of the semiquinone of α -tocopherol in vitro and have suggested that the activity of vitamin E in vivo may be due to this reaction. It is of course possible that the vitamin E activity of the quinone and hydroquinone is due to the conversion of these substances to α -tocopherol in the body. However, in view of our results it is also possible that α -tocopherol is converted to the hydroquinone either directly, or by way of the

⁴ To eliminate the trauma produced by the daily injection of 10 per cent ethanol in propylene glycol, a suspension of the hydroquinone in a 10 per cent aqueous solution of propylene glycol was used in this experiment.

quinone, or its biologically potent isomer which recently has been isolated by Boyer (17), and that the hydroquinone and its semiquinone form a biologically active oxidation-reduction pair.

Finally, and more probably, all of the compounds discussed above may be active in the body. Not one, but several oxidation-reduction pairs may exist, each of which fulfils a biochemical function. The elucidation of this problem depends in a large measure on the development of sensitive and specific methods for the determination of α -tocopherol and of its quinone and hydroquinone in biological materials.

SUMMARY

 α -Tocopherylhydroquinone and α -tocopherylquinone prevent and cure the creatinuria, paralysis, and loss of weight caused by vitamin E deficiency in the rabbit. It may be concluded, therefore, that these compounds possess vitamin E activity.

In bioassays based on the duration of the drop in creatinuria in dystrophic rabbits, the hydroquinone was found to be more active than the quinone. Both compounds were more active when injected intravenously than when given by stomach tube.

At the 5 mg, level the hydroquinone administered intravenously was approximately as active as α -tocopherol. However, in contrast to the results obtained with α -tocopherol, increasing the amount injected did not prolong the duration of the response. Apparently the rabbit's ability to store the hydroquinone is limited, and excess amounts are either destroyed or excreted.

The implications of these results with special reference to the participation of vitamin E in an oxidation-reduction system in the body are discussed.

It is a pleasure to express our appreciation to Dr. Cosmo G. Mackenzie for his advice and help during the course of this study. The authors wish to acknowledge the technical assistance of Mrs. Paula V. Schroeder.

BIBLIOGRAPHY

- Milhorat, A. T., Mackenzie, J. B., Ulick, S., Rosenkrantz, H., and Bartels, W. E., Ann. New York Acad. Sc., 52, 334 (1949).
- Karrer, P., Escher, R., Fritzche, H., Keller, H., Ringier, B. H., and Salomon, H., Helv. chim. acta, 21, 939 (1938).
- 3. John, W., Dietzel, E., and Emte, W., Z. physiol. Chem., 257, 173 (1939).
- 4. Wright, M. D., and Drummond, J. C., Biochem. J., 34, 32 (1940).
- 5. Karrer, P., and Geiger, A., Helv. chim. acta, 23, 455 (1940).
- 6. Golumbic, C., and Mattill, H. A., J. Biol. Chem., 134, 535 (1940).
- 7. Golumbic, C., and Mattill, H. A., J. Biol. Chem., 135, 339 (1940).
- Evans, H. M., Emerson, O. H., and Emerson, G. A., J. Biol. Chem., 113, 319 (1936); 131, 409 (1939).
- 9. Tishler, M., and Evans, H. M., J Biol. Chem., 139, 241 (1941).

- 10. Mackenzie, C. G., and McCollum, E. V., J. Nutr., 19, 345 (1940).
- 11. Tishler, M., and Wendler, N. L., J. Am. Chem. Soc., 63, 1532 (1941).
- 12. Emmerie, A., and Engel, C., Rec. trav. chim. Pays-Bas, 57, 1351 (1938).
- 13. Goettsch, M., and Pappenheimer, A. M., J. Exp. Med., 54, 145 (1931).
- 14. Mackenzie, C. G., Federation Proc., 1, 190 (1942).
- 15. Mackenzie, C. G., Levine, M. D., and McCollum, E. V., J. Nutr., 20, 399 (1940).
- 16. Michaelis, L., and Wollman, S. H., Science, 109, 313 (1949).
- 17. Boyer, P. D., Federation Proc., 8, 187 (1949).

METHEMALBUMIN

INTERACTION BETWEEN HUMAN SERUM ALBUMIN AND FERRIPROTOPORPHYRIN IX*

BY MORRIS ROSENFELD AND DOUGLAS M. SURGENOR

(From the Department of Pharmacology and Experimental Therapeutics, School of Medicine, The Johns Hopkins University, Baltimore, and the Department of Physical Chemistry, Harvard Medical School, Boston)

(Received for publication, September 2, 1949)

Methemalbumin is an abnormal component of blood plasma in certain diseases associated with excessive hemolysis (1). It was first identified by Fairley (2) as a complex of serum albumin and ferriprotoporphyrin. The nature of the protein-heme binding in this pigment has received little study, though in the reduced form of the complex Keilin (3) has postulated linkage with the porphyrin and not with the iron. Methemalbumin is not known to function in catalysis or in oxygen transport, as do certain other heme proteins, and no physiological rôle has been assigned to it. Formation of the complex removes free hematin from the plasma and provides a mechanism for its transport in the blood stream.

The studies reported here were made to characterize further the interaction between crystallized human serum albumin and ferriprotoporphyrin, leading to the formation of methemalbumin. This system is of interest as an association reaction between metalloporphyrin and a coordinating substance such as has been studied by Clark, Taylor, Davies, and Vestling (4) and by Shack and Clark (5).

Materials and Methods

The crystallized human serum albumin used in these studies was kindly supplied by Dr. W. L. Hughes, Jr. Three different preparations were

* At The Johns Hopkins University, this work was supported by a grant-in-aid to one of us (M. R.) from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council. At the Department of Physical Chemistry, Harvard Medical School, this work was originally supported by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institutes of Health. This paper is No. 84 in the series "Studies on the plasma proteins" from the Harvard Medical School, Boston, on the products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. A preliminary report of this work has been presented in Federation Proc., 8, 329 (1949).

used: albumin crystallized with the aid of chloroform (No. 179, recrystallized five times); albumin crystallized with aid of decanol (decanol 10, recrystallized twice (6)); and albumin crystallized as the mercury salt (No. 35 Hg, recrystallized five times (7)). Except when noted, the protein was dissolved in phosphate buffer of ionic strength 0.3 at pH 7.3. Albumin concentrations were based on micro-Kjeldahl analyses with a nitrogen factor of 6.25.

Ferriprotoporphyrin—Commercial crystallized hemin (ferriprotoporphyrin IX chloride) was purified by three further crystallizations from pyridine and chloroform according to the procedure of Hans Fischer (8). On standing in alkaline solution, the hematin was transformed into a compound which, although having an absorption spectrum similar to that of hematin, did not interact with albumin. It was therefore necessary to examine the conditions of stability of ferriprotoporphyrin with respect to this alkaline conversion product. Ferriprotoporphyrin could be separated from the non-binding compound by differences in solubility in the two-phase system: n-butyl alcohol and 0.1 m buffer, either borate or bicarbonate, pH 9.6. The ferriprotoporphyrin was distributed preferentially in the organic phase, whereas the impurity remained in the aqueous phase. After several washings of the organic phase with fresh buffer, pH 9.6, the purified ferriprotoporphyrin was returned to aqueous solution by adding an equal volume of chloroform to the butyl alcohol layer and extracting with 0.1 N (or 0.01 N) sodium hydroxide.

The results of a Craig type counter-current distribution (9) experiment are illustrated in Fig. 1. The ratio of the partition coefficients of the two solutes was so great that essentially complete separation was achieved in an eight plate transfer. The concentration of hematin initially dissolved in the buffer was kept at 250 mg. per liter or less. The partition coefficients varied with the concentration, and less complete separation was achieved at higher concentrations.

With this technique, it was found that 25 to 30 per cent of the inactive product was formed in a solution of ferriprotoporphyrin in 0.1 m borate buffer at pH 9.6 during a 3 hour period. In 0.1 n sodium hydroxide for 1 hour, 15 per cent conversion product was formed, while in 0.01 n sodium hydroxide only 7 per cent impurity was formed in the same time. For these studies, hemin was quickly dissolved in 0.1 n sodium hydroxide and was immediately diluted to a sodium hydroxide concentration of 0.001 n. Solutions of hematin containing no more than 5 per cent of the conversion product were prepared in this manner. The impurity did not measurably increase during several hours.

Titrations were carried out by diluting 1 part of the protein solution with 2 parts of the 0.001 N sodium hydroxide solution of hematin, resulting in an ionic strength of 0.1 and pH 7.4. The solutions were maintained at 38° for $\frac{1}{2}$ hour to assure equilibrium, after which they appeared to remain

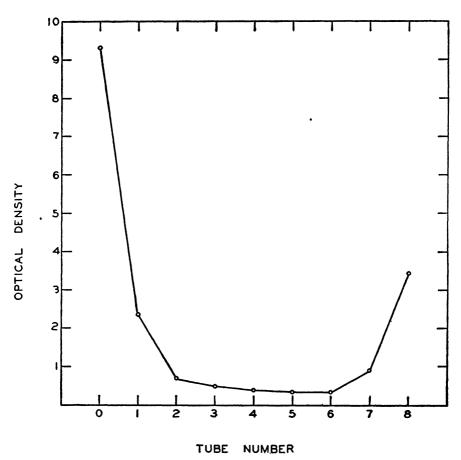


Fig. 1. Counter-current distribution pattern of a mixture of ferriprotoporphyrin and non-binding conversion product. Two phase solvent system; n-butyl alcohol and 0.1 m borate buffer, pH 9.6, in equal volumes. Tube 0 contained initially 250 mg. per liter of hemin which had stood 3 hours in the borate buffer. Optical densities at 403 m μ , 1 cm. path.

stable for 24 hours or more. This stabilizing effect of protein was observed even in the presence of a large molar excess of ferriprotoporphyrin over that which could be bound by the protein; *i.e.*, 10 moles of ferriprotoporphyrin per mole of albumin. Hematin was diluted in this way with buffer

in the absence of protein as a blank. Readings were made immediately because ferriprotoporphyrin is unstable at pH 7.4 as a result of association and formation of aggregates. Optical density readings were not, as a rule, carried out at protein concentrations less than 0.1 mole per mole of ferriporphyrin, owing to the instability of the ferriporphyrin.

Titrations were carried out either in a series of tubes containing varying amounts of reagents read separately in the spectrophotometer, or by the use of a Gilmont type of ultramicro burette. In the latter case, successive amounts of hematin solution were titrated directly into the buffered (pH 7.4, $\mu = 0.1$) albumin solution in a thermostat-controlled (38°) spectrophotometer cell, and the optical density was read a few minutes after each addition. This method had the advantage of requiring very small amounts of protein for a complete titration curve. It had the disadvantage that the hematin could have varied slightly with time.

Spectrophotometric measurements were carried out in a Beckman model DU spectrophotometer.

Results

Evidence of interaction with crystallized human serum albumin was obtained from the absorption spectra of ferriprotoporphyrin in the presence and absence of protein. These are shown in Fig. 2. In the presence of an equimolar amount of albumin, the broad absorption maximum of free ferriprotoporphyrin at 380 to 390 m μ (ϵ (385 m μ) = 4.8 × 104) was replaced by the narrow band of methemalbumin with maximum absorption at 403 m μ (ϵ (403 m μ) = 8.3 × 104). The protein-ferriporphyrin complex had absorption maxima at 500, 540, and 623 m μ . Ferriprotoporphyrin showed no discrete bands in the visible region between 400 and 650 m μ at pH 7.4, although in 0.1 N sodium hydroxide solution a band appeared at 610 m μ .

Dialysis experiments, which have proved useful in many studies of this kind, yielded only incomplete information, since it has been shown (5) from ultracentrifugal and diffusion measurements that ferriprotoporphyrin associates in aqueous solution to form aggregates of particle weight between 20,000 and 60,000. In an alkaline reaction in which association was diminished, conversion to the inactive form occurred. Since lengthy dialysis was necessary, these difficulties made it impossible to achieve equilibrium with respect to the ferriporphyrin. Experiments indicated, however, that the rate of diffusion of iron porphyrin from methemalbumin to protein-free buffer was very much slower than the rate of diffusion of free ferriprotoporphyrin to albumin.

Spectrophotometric Titrations—The most accurate and direct method of obtaining quantitative data appeared to be by direct titration of one re-

¹ We have used the 0.1 ml. Gilmont ultramicro burette manufactured by the Emil Greiner Company.

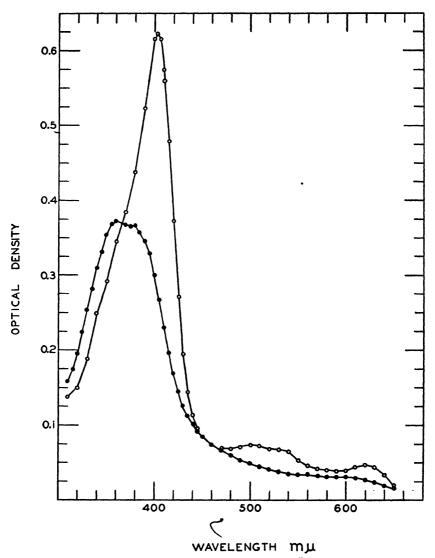


Fig. 2. Spectrophotometric curves of methamalbumin and ferriprotoporphyrin. O, methemalbumin formed by combining ferriprotoporphyrin (5 mg. per liter as hemin, 7.7×10^{-6} m) and crystalline human serum albumin (0.53 gm. per liter, 7.7×10^{-6} m). •, ferriprotoporphyrin, 5 mg. per liter as hemin. pH 7.4 phosphate buffer of ionic strength 0.1. Optical path, 1 cm.

actant with the other. To assure reproducibility, some measurements were made on the rate of reaction of ferriprotoporphyrin with albumin. Solutions of protein and ferriprotoporphyrin, at the desired temperature,

were rapidly mixed in amounts to give a final albumin concentration of 2.1 gm. per liter and a hemin concentration of 10 mg. per liter in a 0.1 n borate buffer at pH 8.2. The molar ratio of albumin to ferriprotoporphyrin was 2:1. The reaction mixture was immediately transferred to the spectrophotometer cell in a cuvette chamber at constant temperature, and the rate of increase in extinction at 403 m μ was measured. The reaction took place rapidly and was 91 per cent complete in 30 minutes at 22° and complete in the same time at 38°. Solutions for measurement were therefore maintained at 38° for 30 minutes. Since the complex formed at 38° was stable at 22°, optical determinations were carried out at room temperature.

The data obtained on titrating a solution of albumin with ferriprotoporphyrin are presented in Fig. 3. The titration curve at a wave-length of 403 m_{\mu} consisted of an initial linear section, an intermediate zone of curvature, and a second linear section. The slope of this latter section, which was constant up to iron porphyrin concentrations in 40-fold molar excess over albumin, appeared to be chiefly due to the appearance of free ferriprotoporphyrin in the solution, and, to a lesser extent, to a non-specific enhancement of the extinction of free ferriporphyrin by protein. shown by the lower two curves in Fig. 3. The titration curve of γ -globulin with ferriprotoporphyrin consisted of a straight line whose slope was nearly the same as the second linear portion of the titration curve of albumin, and slightly greater than the slope for free ferriprotoporphyrin alone. A possible explanation of this non-specific effect is that both γ -globulin and albumin bound ferriprotoporphyrin to yield a product characterized by a low binding constant, or that the protein produced a change in the state of physical dispersion of iron porphyrin aggregates. In the case of γ -globulin, this appeared to be the only reaction; in the case of albumin it appeared to be secondary, and of minor extent in comparison to the primary interactions which were characterized by high binding constants and by a pronounced change in spectral absorption. Subtraction of the ferriprotoporphyrin reference line, corrected for the non-specific effect of protein, from the albumin titration curve (Fig. 4, upper graph) almost completely eliminated the positive slope of the second linear section of the curve.

The addition of ferriprotoporphyrin to albumin resulted in a linear increase in the optical density at 403 m μ (Figs. 3 and 4) until 1 mole of iron porphyrin per mole of albumin had been added. There followed a zone of curvature which extended to the addition of 2 moles of iron porphyrin. Beyond this end-point there was an accumulation of free ferriprotoporphyrin in solution. The over-all reaction can be represented as follows:

$$L + 2M \to LMM \tag{1}$$

The molecular extinction coefficient of the complex LMM (2 moles of

ferriprotoporphyrin per mole of albumin), as determined from these data, was taken as $\epsilon_{LMM} = 15.0 \times 10^4$ (403 m μ).

When the spectrophotometric titration of albumin with ferriprotoporphyrin was measured at 623 m μ , the data indicated the binding of 4 moles of iron porphyrin per mole of albumin. Since this was not reflected in meas-

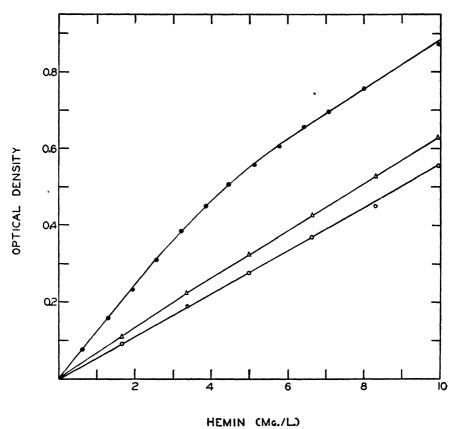
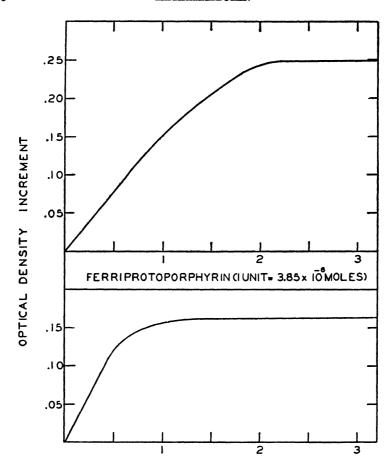


Fig. 3. Titration of crystalline human serum albumin and γ -globulin with ferriprotoporphyrin. •, titration of serum albumin (0.266 gm. per liter, 3.85×10^{-6} M). The solid line was computed from equations. The curve has been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity. Δ , titration of γ -globulin, fraction II-1,2, 0.266 gm. per liter. \bigcirc , ferriprotoporphyrin reference line. Wave-length 403 m μ , 1 cm. path.

urements at 403 m μ , the iron porphyrin bound after the first 2 moles appeared not to affect the Soret band (403 m μ) sufficiently to reveal the addition. The additional 2 molecules of ferriporphyrin may have interacted with the protein by a different mechanism from that which governed



SERUM ALBUMIN (IUNIT = 3.85 × 10 MOLES)

Fig. 4. Corrected titration curves. The curves represent the increment in optical density due to formation of methemalbumin (LM and LMM). The titration data have been corrected for absorption due to protein, ferriprotoporphyrin, and to the non-specific effect of protein. The curves are for ferriporphyrin of 100 per cent purity. Upper graph, titration of serum albumin (3.85×10^{-6} mole per liter) with ferriprotoporphyrin. Lower graph, titration of ferriprotoporphyrin (3.85×10^{-6} mole per liter) with serum albumin. Wave-length 403 m μ , 1 cm. path.

the binding of the first 2 molecules. This, however, would be an *ad hoc* assumption. In dealing with solutions of ferriporphyrin there is always the uncertainty that changes in state of aggregation may alter the absorption. Thus, what we have called the non-specific effect of γ -globulin was perhaps due, in part, to change in degree of dispersion of ferriporphyrin

by this protein. In the absence of conclusive evidence we have tentatively treated the data on the simplest theoretical basis.

The converse type of titration in which serum albumin was added stepwise to a fixed amount of ferriprotoporphyrin is illustrated in Fig. 5. The end-point occurred when 1 mole of albumin had been added per mole of

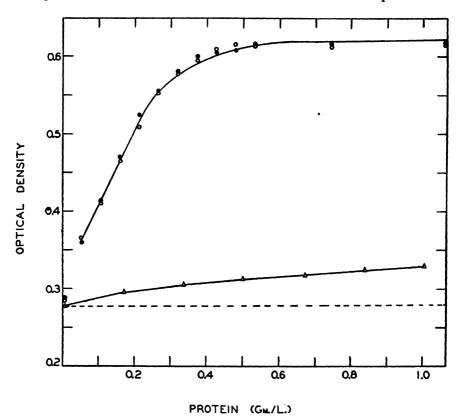


Fig. 5. Titration of ferriprotoporphyrin with crystalline serum albumin and with γ -globulin. Concentration of ferriporphyrin, 5 mg. per liter as hemin (7.7 \times 10⁻⁶ M). O and •, titration with serum albumin. Solid line computed from equations. The curve has been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity. Δ , titration with γ -globulin, fraction II-1,2. Wave-length 403 m μ , 1 cm. path.

ferriprotoporphyrin (Fig. 4, lower graph, and Fig. 5). Accordingly, in the presence of excess albumin, the reaction was

$$L + M \to LM \tag{2}$$

Beyond the end-point, further increase in optical density could not be

detected provided a slight increment was corrected by subtraction of a small protein blank. From these titration curves, ϵ_{LM} (403 m μ) has been taken as 8.3×10^4 .

Influence of pH, Buffer Ions, and Ionic Strength—Methemalbumin (LM) was first formed by mixing ferriprotoporphyrin with albumin in an unbuffered 0.15 m sodium chloride solution. Buffer was then added, and the solutions were incubated another 15 minutes to permit reequilibration. The results of such an experiment are shown in Fig. 6. The two buffers, acetate and phthalate, were adjusted to give an ionic strength of 0.15 throughout the pH range. Isoelectric precipitation and denaturation of protein invalidated readings between pH 3.0 and 4.6. There was progressive increase in optical density from pH 4.6 to 7.0. In this pH range.

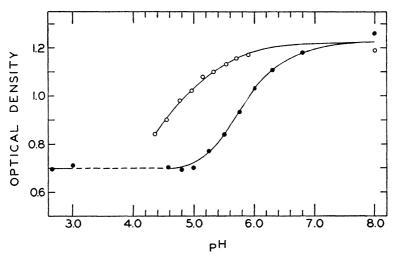
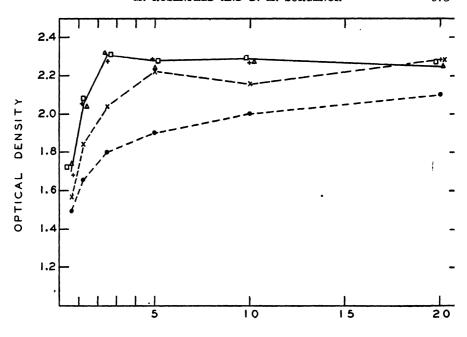


Fig. 6. Optical density of methemalbumin in relation to pH. O, acetate buffer;

•, phthalate buffer. Ionic strength 0.15.

density readings were higher in acetate than in phthalate, suggesting a specific ion effect. However, the two curves converged at pH 7.0 and remained constant between pH 7.0 and 10.0.

In another type of experiment, Fig. 7, ferriprotoporphyrin was titrated with protein in buffers of different composition and pH. Identical curves were obtained in phosphate, borate, and glycine buffers at pH 7.5, 9.0, and 9.9 respectively. Maximal optical density values, corresponding to complete binding, were reached when an equimolar amount of albumin was added to the ferriporphyrin. At more acid reactions, namely at pH 5.0 and 6.0 in acetate and phosphate buffers, the complex dissociated and complete binding of the ferriporphyrin was not attained until protein was



SERUM ALBUMIN GM./L.

Fig. 7. Titration of ferriprotoporphyrin with serum albumin at various pH levels. Ferriporphyrin concentration, 20 mg. per liter as hemin (30.7 \times 10⁻⁶ M). Fraction V, containing 95 per cent albumin, used in the titrations. +, glycine, pH 9.9; \square , borate, pH 9.0; \triangle , phosphate, pH 7.5; \times , phosphate, pH 6.0; \bullet , acetate, pH 5.0.

Table I

Effect of Concentration on Dissociation of Methemalbumin at pH 7.4 and Ionic

Strength 0.1

Concentration, moles per liter × 106, as albumin

					3.85*	0.385	0.077
Extin	ction p	er cm.	at 403 mµ		0.551	0.0523†	0.0102†
				dilution		0.523	0.510
"		" "	calculated (I	Equations 7 and 8)	0.555	0.537	0.520
Mole	fractio	n of fe	rriprotoporph	M yrin as M	0.03	0.11	0.23
"	"	"	î û î	" LM	0.04	0.10	0.17
66	"	"	"	" LMM	0.93	0.79	0.60

^{*} Dilutions were made of this solution which had a composition of 2 moles of ferriprotoporphyrin per mole of albumin. The calculated extinctions have been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity.

[†] These solutions were measured in a 10.0 cm. cuvette.

added in large excess. The acid dissociation was reversed when the pH was adjusted to 7.0 or above by the addition of an alkaline buffer.

When ferriprotoporphyrin was added to albumin solutions in phosphate buffers of different ionic strength at pH 7.4, differences in end-point were not observed when the ionic strength varied from 0.03 to 0.30.

These experiments suggest that methemalbumin formation varied with the pH and with the specific composition of the buffers from pH 4.5 to 7.0. From pH 7.0 to 10.0, however, maximal binding appears to have occurred and to have been independent of pH and ionic strength.

Influence of Concentration—When a solution of methemalbumin was diluted, it failed to obey Beer's law. The deviation suggested the dissociation of the complex LMM in dilute solution to yield increased amounts of the species LM and M. The results are presented in Table I. The calculated extinctions and mole fraction of ferriprotoporphyrin as M, LM, and LMM at the given concentrations have been obtained by use of the equations and the intrinsic association constant, K_0 , which are discussed below.

DISCUSSION

Since the experimental methods used did not permit direct measurement of the molecular concentrations of the various species involved in the equilibrium, experimental equilibrium constants could not be calculated. In an attempt to assess the magnitude of the constants, we have made use of equations applicable to such equilibria. This treatment has been used for the dissociation of carbonic acid and is similar to that used by Michaelis and Schubert (10) for a two-step oxidation-reduction, and by Shack and Clark (5) for metalloporphyrin coordination.

Using the symbols of Clark, with L representing the ligand, serum albumin, and M the metalloporphyrin, one obtains two equilibrium constants, Equations 3 and 4, for the reactions expressed by Equations 1 and 2.

$$\frac{[LM]}{[L] \times [M]} = K_1 \tag{3}$$

$$\frac{[LMM]}{[LM] \times [M]} = K_s \tag{4}$$

At any point in a titration, the total concentration of serum albumin [1] whether free or combined, and of ferriprotoporphyrin [m] in all forms, is given by the expressions

$$[1] = [L] + [LM] + [LMM]$$
 (5)

$$[m] = [M] + [LM] + 2[LMM]$$
 (6)

By combining the four equations, Nos. 3 to 6, and introducing the mo-

lecular extinction coefficient (ϵ) of the various absorbing species, one arrives at a pair of equations, Nos. 7 and 8, which serve to define the optical density, D, of a solution, 1 cm. in depth, as a function of the concentration of ferriprotoporphyrin added at each step of the titration.

$$D = \frac{\epsilon_L + \epsilon_{LM} K_1[M] + \epsilon_{LMM} K_1 K_2[M]^2}{1 + K_1[M] + K_1 K_2[M]^2} \times [1] + \epsilon_M[M]$$
 (7)

$$[...] = \frac{K_1[M] + 2K_1K_2[M]^2}{1 + K_1[M] + K_1K_2[M]^2} \times [1] + [M]$$
(8)

In the above equations, ϵ_L was zero, since a protein blank was used. ϵ_M varied slightly with protein concentration, owing to the non-specific effect of protein. In the absence of protein, $\epsilon_M = 3.65 \times 10^4$ (403 m μ); at 0.05 gm. of albumin per liter, $\epsilon_M = 3.9 \times 10^4$; and at 0.50 gm. of albumin per liter, $\epsilon_M = 4.2 \times 10^4$. Interpolation was used for intermediate values. The experimentally determined values for ϵ_{LM} and ϵ_{LMM} were used; namely, $\epsilon_{LM} = 8.3 \times 10^4$ and $\epsilon_{LMM} = 15.0 \times 10^4$.

In a recent discussion of the interaction of small molecules and ions with proteins, Scatchard (11) has shown that the experimental results can often be described by assuming that the protein possesses a maximum of n sites which bind the small molecule. In many instances, all n points can be assumed to have the same intrinsic association constant. The formation of any given protein-small molecular compound, in which the number of small molecules is generally less than n, will be described by this constant, modified by statistical and electrostatic factors. To describe the interaction of albumin with ferriprotoporphyrin we have made two assumptions: that n=2 and that the reaction at either point is independent of combination at the other, except for statistical effects. Then, if we denote the intrinsic association constant by K_0 , the actual equilibrium constants K_1 and K_2 in the equations above are related to K_0 as follows:

$$K_1 = \frac{n}{1} K_0 = 2K_0 \tag{9}$$

$$K_2 = \frac{n-1}{2} \times K_0 = \frac{1}{2}K_0 \tag{10}$$

$$\frac{K_1}{K_2} = 4 \tag{11}$$

The results obtained from the titrations at a wave-length of 623 m μ and the interaction of ferriprotoporphyrin with protein which we have termed non-specific may indicate that there are other sites on the albumin molecule which react with ferriprotoporphyrin. However, the formation of these higher compounds had little or no effect upon spectral absorption at the

 $403 \text{ m}\mu$ wave-length, and the association constants for their formation must be much smaller than that for the addition of the first two groups. Therefore, the formation of such higher complexes should not materially invalidate the assumption that a single intrinsic association constant can be used for the first two groups.

Based on the above considerations, the attempt was made to choose an intrinsic association constant by the construction of a set of titration curves. Good agreement with experimental data was obtained by use of the single association constant $K_0 = 10^8$, where $K_1 = 2 \times 10^8$ and $K_2 = 5 \times 10^7$. The titration curves so obtained are represented in Figs. 3 and 5. The calculated extinctions at the three levels of concentration shown in Table I also agreed well with the experimental values. Since dilution mainly affected the second equilibrium, these results tend to substantiate the value assigned to K_2 .

While the binding of ferriprotoporphyrin by albumin can be satisfied by a single intrinsic association constant, K_0 , according to the above treatment, it should be emphasized that this is not an experimentally derived constant. Indeed, good agreement was also obtained when $K_1 = 10^9$ and $K_2 = 10^7$ ($K_1/K_2 = 100$). It is, therefore, not possible to make any deductions from these data as to the extent of interaction, if there is any, between the two heme groups on the albumin molecule.

A large body of previous data (5) indicates that ferriprotoporphyrin exists in solution as large micelles within which there are dimeric units. Although in the development of our equations the metalloporphyrin has been treated as a monomeric species, exactly the same titration curves are obtained on the basis of dimeric units. The equations merely indicate the molecular proportions of the various components and do not reveal the degree of polymerization.

No observable differences in behavior toward ferriporphyrin were found with the several lots of human serum albumin used. Albumin alone, of the plasma proteins studied, interacted with ferriprotoporphyrin in this way. Preliminary observations with crystalline bovine serum albumin suggested that its ferriprotoporphyrin complex was different in spectral characteristics from the complex with human serum albumin. The studies with other plasma proteins and a method for the quantitative determination of albumin based on the present investigation will soon be reported.

This study supports the validity of measurements previously reported (1) on plasma methemalbumin levels during the administration of certain drugs. Assuming an average of 35 gm. of serum albumin per liter of plasma, over 300 mg. of hemin per liter of plasma could be carried in the form of the 1:1 complex. The highest levels encountered were 25 mg. per liter, and this would combine with less than 10 per cent of the serum al-

bumin. Under such conditions, all the ferriprotoporphyrin would be bound. Furthermore, dilution of methemalbumin-containing plasma for measurement was never greater than 5-fold, and this would have resulted in no appreciable dissociation of the complex.

We thank Miss Elizabeth O. Burr for assistance in these measurements.

SUMMARY

Spectrophotometric titration at the 403 m μ absorption band of methemalbumin indicated that human serum albumin reacts with ferriprotoporphyrin IX to form protein-heme complexes in the molar ratios of 1:1 and 1:2. By use of the mass law equations, association constants have been chosen which give good agreement with the experimental results.

Reversible dissociation of the complex occurred between pH 7.0 and 4.6; between pH 7.0 and 10.0 the reaction was not affected by pH, specific ion effects, or moderate changes in ionic strength. The reaction was accelerated at 38° and equilibrium was established within $\frac{1}{2}$ hour at this temperature.

BIBLIOGRAPHY

- Rosenfeld, M., Zubrod, C. G., Blake, W. D., and Shannon, J. A., J. Clin. Invest., 27, pt. 2, 138 (1948).
- 2. Fairley, N. H., Quart. J. Med., 10, 115 (1941).
- 3. Keilin, J., Nature, 154, 120 (1944).
- Clark, W. M., Taylor, J. F., Davies, T. H., and Vestling, C. S., J. Biol. Chem., 135, 543 (1940).
- 5. Shack, J., and Clark, W. M., J. Biol. Chem., 171, 143 (1947).
- Cohn, E. J., Hughes, W. L., Jr., and Weare, J. H., J. Am. Chem. Soc., 69, 1753 (1947).
- 7. Hughes, W. L., Jr., J. Am. Chem. Soc., 69, 1836 (1947).
- 8. Fischer, H., Org. Syntheses, 21, 53 (1941).
- 9. Craig, L. C., J. Biol. Chem., 155, 519 (1944).
- 10. Michaelis, L., and Schubert, M. P., Chem. Rev., 22, 437 (1938).
- 11. Scatchard, G., Ann. New York Acad. Sc., 51, 660 (1949).

BIOCHEMICAL STUDIES ON CHLORAMPHENICOL (CHLOROMYCETIN*)

III. ISOLATION AND IDENTIFICATION OF METABOLIC PRODUCTS IN URINE†

By ANTHONY J. GLAZKO, WESLEY A. DILL, AND MILDRED C. REBSTOCK

(From the Research Laboratories, Parke, Davis and Company, Detroit)

(Received for publication, October 29, 1949)

The antibiotic chloramphenical (Chloromycetin), first isolated from cultures of *Streptomyces venezuelae* (1), has been identified as p(-)-threo-1-p-nitrophenyl-2-dichloracetamido-1,3-propanediol (2). The structure of this compound is shown below:

When chloramphenicol is administered by mouth to normal human subjects, only 5 to 10 per cent of the administered dose is excreted unchanged in the urine, as determined by microbiological assay methods (3, 4). However, with colorimetric methods for aromatic nitro compounds (5), from 75 to 90 per cent of the administered dose is accounted for in 24 hour urinary excretion in man (3). The major portion of these nitro compounds exhibits no antibiotic activity, and represents inactive degradation products of chloramphenicol. In lower animals, lesser amounts of nitro compounds are recovered in the urine, the remainder being excreted in the bile and feces, where considerable reduction occurs with the formation of aromatic amines (3).

The present communication is concerned with the isolation and identification of chloramphenical and its metabolic products from urine, chromatographic and counter-current methods for separation being used. The major excretory product in urine is shown to be a monoglucuronide derivative of chloramphenical by enzymatic and chemical degradation studies.

^{*} Parke, Davis and Company's trade name for chloramphenicol.

[†] Reported in part at the thirty-third annual meeting of the Federation of American Societies for Experimental Biology, Detroit, April (1949).

Procedure

Aromatic nitro compounds are determined colorimetrically by reduction with titanous chloride, followed by diazotization and coupling with the Bratton-Marshall reagent (5). The results are expressed as "chloramphenicol equivalents," since metabolic products of chloramphenicol containing the nitro group, but having no antibiotic effect, are included in the analysis. Chloramphenicol is usually determined by microbiological assay with *Shigella sonnei* by using a turbidimetric procedure (6), although the colorimetric method is also satisfactory when the antibiotic is first separated from inactive nitro compounds by extraction with organic solvents (5).

Paper strip partition chromatography was conducted according to the methods described by Consden, Gordon, and Martin (7). The materials to be chromatographed were first concentrated in a narrow band on the paper strip (Schleicher-Schüll No. 112) by use of the hot wire technique described elsewhere (8). After chromatography the position of the nitro compounds on the strips was established by the formation of colored derivatives. With a fine atomizer a 1:10 dilution of the stock 15 per cent titanous chloride reagent in 1 n HCl is sprayed onto the paper strip. While still damp, the strip is lowered into a glass cylinder containing a smal amount of liquid bromine at the bottom, and the excess titanous reagent is rapidly oxidized by the bromine vapor, eliminating any interference with subsequent reactions. The treatment with bromine seems to affect the reduced compounds directly, since the carmine color resulting from this procedure is quite different from the amethyst color produced in the usua colorimetric procedure without bromination (5). The strip is then aired in a hood to remove the free bromine. After a few minutes, the strip is sprayed with a mixture of 10 parts butyl nitrite, 5 parts n-butanol, and : part glacial acetic acid. After standing 2 minutes, the strip is sprayed with a 0.1 per cent solution of N-(1-naphthyl)ethylenediamine dihydro chloride in a mixture of 95 ml. of n-butanol and 5 ml. of 2 n HCl. A pinl color indicates the presence of aromatic nitro compounds. A control fo aromatic amines is also run by spraying the control strip with butyl nitrit and the coupling reagent, but omitting the reduction and bromination steps.

EXPERIMENTAL AND DISCUSSION

Counter-Current Partition of Nitro Compounds in Rat Urine—Since the administration of chloramphenical has been shown to result in the excretion of more than one aromatic nitro compound in the urine (3), an at tempt was made to fractionate these compounds by the counter-current extraction procedures described by Craig (9). A 24 plate apparatus was

used in these experiments. The solvents selected gave approximately equal distribution of chloramphenicol between equal volumes of the two immiscible phases at 24°. These consisted of 0.2 m phosphate buffer at pH 6.7 and a mixture of 79 per cent heptane and 21 per cent isoamyl alcohol. The aqueous and organic phases were mutually saturated by shaking together before use.

Urine collected from a series of rats over a 17 hour period following subcutaneous administration of 100 mg. of chloramphenicol per kilo, body weight, was diluted with an equal volume of 0.4 m phosphate buffer at pH 6.7. 8 ml. of this mixture were extracted in the 24 plate Craig apparatus against 8 ml. of the heptane-isoamyl alcohol mixture described above. The aqueous and organic phase in each plate was then analyzed for aro-

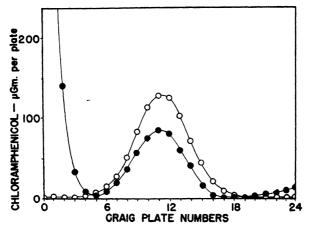


Fig. 1. Counter-current extraction of rat urine to demonstrate the presence of chloramphenicol. O, obtained with a known sample of chloramphenicol; •, obtained with rat urine. The water-soluble end of the series is in the lower plate numbers.

matic nitro compounds in terms of chloramphenicol equivalents by ultraviolet absorption at 278 m μ . A parallel series was set up in the same manner by using a known sample of chloramphenicol. The results are presented in Fig. 1.

From these data it is evident that a high concentration of a water-soluble nitro compound is present in the urine. A second component in the middle of the series (with peak concentration at Plate 11) corresponds in distribution with the known sample of chloramphenicol. The substance in Plate 11 showed the same maximum ultraviolet absorption as observed with chloramphenicol (278 m μ), further establishing its identity.

Isolation of Chloramphenical from Human Urine-The presence of un-

changed chloramphenicol in human urine was definitely established by direct isolation and crystallization of the antibiotic. Urine specimens were obtained from patients undergoing treatment with chloramphenicol for bacillary infections of the urinary tract. Approximately 4 liters of urine at pH 5 were extracted with 1500 ml. of ethyl acetate, and extracted a second time with 1000 ml. of the organic solvent. The ethyl acetate extracts were pooled and evaporated to dryness under reduced pressure at room temperature. The residue was further purified by an 8 plate countercurrent extraction with 0.2 m phosphate buffer at pH 6.7 and a mixture of 79 per cent heptane and 21 per cent isoamyl alcohol. This extraction procedure was carried out in 500 ml. glass-stoppered Pyrex bottles, with 150 ml. volumes of phosphate buffer and the heptane-isoamyl alcohol mixture described above. The solutions in Plates 3, 4, and 5, containing most of the chloramphenical, were pooled and concentrated by evaporation. The residue was extracted with three separate portions of ethyl acetate, the pooled extracts were evaporated to dryness, and the residue was redissolved in chloroform. On the addition of 6 volumes of petroleum ether a heavy precipitate appeared. The precipitation process from chloroform was repeated several times and the final product was dried in an oven at 100°. This preparation gave a melting point of 151.0° as compared with 151.5°, for a known sample of chloramphenical (uncorrected readings), and produced no depression in melting point when mixed with chloramphenicol. Maximum ultraviolet absorption was found at 278 mµ, and the $E_{1 \text{ cm}}^{1\%}$ value was found to be 290 in 0.1 N HCl and 293 in pH 6.6 phosphate buffer, compared with values of 290 to 297 obtained with known samples of chloramphenicol. When assayed for antibiotic activity, this preparation was found to possess 98 to 100 per cent of the activity of a known sample of chloramphenicol.

Paper Strip Chromatography of Urine—Paper strip partition chromatography was used to study the metabolic products of chloramphenicol in urine. The solvent employed was a mixture of n-butanol, with 2.5 per cent phenol and 2 per cent pyridine added. Nitro compounds were detected by the color reaction already described ("Procedure"). Typical data obtained with urine specimens after administration of chloramphenicol are presented in Table I. Urine from man, dog, and rat showed the presence of three principal aromatic nitro compounds. These were identified by comparing the R_F values with known derivatives of chloramphenicol and appeared to be the same in all the species studied.

Band I was found to be identical with that produced by the addition of chloramphenicol to normal urine. It represents the small amount of antibiotic excreted in the urine following the administration of this drug, whose isolation and chemical identity with chloramphenicol were established in the preceding section.

Band II was found to correspond with that produced by the addition of p(-)-threo-1-p-nitrophenyl-2-amino-1,3-propanediol to normal urine. This compound is readily obtained by acid or alkaline hydrolysis of chloramphenical at the amide linkage with the formation of dichloroacetic acid and the optically active base (2). Its structure is given below:

$$\begin{array}{c|c} H & NH_2 \\ & | & | \\ C-C-C+CH_2OH \\ & | & | \\ OH & H \end{array}$$

$$(II)$$

In all urine specimens studied Band III was most prominent, giving the greatest color intensity and covering the greatest area. The band due

Table I

Chromatography of Nitro Compounds in Urine Following Administration

of Chloramphenicol

Urine samples were chromatographed on paper strips by the descending technique, and the nitro compounds were detected by the color reaction described in the text. In each experiment known samples of chloramphenicol, the base resulting from the hydrolysis of chloramphenicol, and chloramphenicol-glucuronide were added to normal urine samples to serve as controls.

Sample chromatographed	Band I	Band II	Band III	
	R _F	R _F	R	
Urine, rat	0.89 - 0.94	0.54-0.60	0.30-0.37	
" dog		0.58-0.63	0.34-0.40	
" man	0.92 - 0.95	0.58-0.65	0.28-0.32	
Chloramphenicol (I)		l		
Base from hydrolyzed chloramphenicol (II)		0.56-0.67		
Chloramphenicol-glucuronide (III)			0.30-0.40	

to chloramphenicol (I) was less marked, while that due to the amino diol hydrolysis product (II) was least distinct. Consequently it would appear that the substance responsible for Band III is the principal metabolic product of chloramphenicol excreted in the urine of man, dog, and rat. The position of Band III indicates that it is more water-soluble than either chloramphenicol or its hydrolysis product, and it probably corresponds with the water-soluble nitro compound which was noted in the counter-current extraction experiment (Fig. 1).

Similar results were obtained with two-dimensional chromatographic methods. In all cases controls were run by the addition of known compounds to the urine specimens. The first solvent employed was *n*-butanol containing 1.5 per cent glacial acetic acid, while the second solvent was

n-butanol with 1.5 per cent ammonium hydroxide used. Colors were developed following reduction with titanous chloride, as already described for the paper strips. By using samples of rat and dog urine collected after parenteral administration of chloramphenicol, three distinct spots were observed, corresponding to Bands I to III on the single dimensional strips. In addition, two other faint spots were observed, one being close to the area due to chloramphenicol and the other close to the substance responsible for Band III.

The chromatographic technique was also applied to the study of nitro compounds in bile collected from dogs and guinea pigs. Although high concentrations of nitro compounds have been observed in bile, the antibiotic activity is usually quite low (3). By using the paper strip partition procedure described above, the predominant nitro compound in bile was found to have the same R_F value as the substance in urine characterized by Band III.

Isolation and Identification of Glucuronide Derivative of Chloramphenicol from Urine—The compound responsible for Band III on the paper strip chromatograms was isolated by the procedure described below, and identified as a monoglucuronide derivative of chloramphenicol.

6 gallons of urine collected from human subjects who were undergoing treatment with chloramphenicol for urinary tract infections were concentrated to 3500 ml. in a vacuum pan at room temperature and filtered. The salt content of the filtrate was decreased by freezing the mixture until an icy slush formed and removing the solids by centrifugation. An equal volume of n-butanol was then added to the urine concentrate (at pH 6.7) and an 8 plate counter-current extraction series was run with Pyrex bottles instead of with the Craig apparatus (9). At the end of the run, petroleum ether was added to each plate equivalent to two-thirds the volume of the organic phase, and on shaking, the nitro compounds were found to be completely transferred to the aqueous phase. Aliquots were analyzed for nitro compounds by the titanium reduction procedure (5), and the highest concentrations were found close to the middle of the series.

The three central plates containing the highest concentrations of nitro compounds were pooled and concentrated in a vacuum pan. The pH was adjusted to 8.0 and the solution cooled in a deep freeze unit to crystallize out excess salts. After readjustment of pH 6.7, the solution was subjected to a second counter-current extraction under the conditions already described. The contents of the central plates were again pooled and concentrated in a vacuum pan. The concentrate was then lyophilized to remove the water and redissolved in absolute ethanol. The addition of petroleum ether resulted in the appearance of a precipitate which was filtered off, washed with excess petroleum ether, and dried. The

material was again dissolved in absolute ethanol and reprecipitated with petroleum ether.

The final preparation was a fine white powder which proved to be extremely hygroscopic, turning brown in the presence of moisture. It was dried to constant weight in vacuo over P_2O_5 in 24 to 48 hours at room temperature. When heated, it decomposed without giving a sharp melting point. The compound was extremely soluble in water, soluble in the lower alcohols, slightly soluble in acetone, and insoluble in ether. Qualitative tests revealed the presence of chlorine and an aromatic nitro group. A faint positive test was obtained for glucuronic acid by the naphthoresorcinol reaction, and infra-red absorption data supported this observation. The ultraviolet absorption in methanol showed a peak at 273 m μ , presumably due to the para-nitro structure, with an $E_1^{1\%}$ of 167. The apparent molecular weight calculated from the ultraviolet absorption data and corrected for volatile loss is 566.

Microanalytical data gave the following per cent composition: C 40.48, 40.37; H 4.48, 4.73; N (Dumas) 5.03, 5.03; total Cl 11.56; Na 4.98; O (by difference) 33.44; ash 8.31. Based on a molecular weight of 566, the following empirical formula is obtained: C₁₉H₂₅N₂Cl₂O₁₂Na. This is in fair agreement with the following structure, representing a monoglucuronide derivative of chloramphenical with one alcohol of crystallization present:

$$O_{2}N \longrightarrow \begin{array}{c|c} H \ HN - CO - CHCl_{2} \\ \hline \\ \\ \\ C - C - CH_{2} - O - C - (CHOH)_{3} - C - COON_{8} \cdot C_{2}H_{5}OH \\ \hline \\ OH \ H \end{array}$$

$$(III)$$

This compound has a molecular weight of 567, being represented by the empirical formula $C_{17}H_{19}N_2O_{11}Cl_2Na\cdot C_2H_6OH$. The theoretical per cent composition is C 40.20; H 4.41; N 4.94; Cl 12.51, Na 4.05; O 33.84, showing good agreement with the experimentally determined composition. The actual point of attachment of glucuronic acid to chloramphenicol was established by the experiments described in another section. The compound appeared to be homogeneous, yielding a single nitro band on paper chromatography and giving a single peak in the center of the distribution series when subjected to a counter-current separation in the 24 plate Craig apparatus between 0.2 m phosphate buffer at pH 6.7 and n-butanol.

Enzymatic Hydrolysis of Chloramphenicol Glucuronide—The compound described in the preceding section showed no antibacterial activity when tested against Shigella sonnei. Its probable structure (III) indicates that

chloramphenicol is in no way altered, except for conjugation with glucuronic acid through a single —OH group. Consequently attempts were made to liberate active chloramphenicol by enzymatic hydrolysis. It was found that more than 50 per cent of the theoretical activity could be regained by incubating this conjugate with minced rat liver or spleen, and that the optimum pH for this hydrolysis was 4.5 to 5.0. Purified preparations of β -glucuronidase gave even better recovery of active antibiotic, often exceeding 70 per cent of the theoretical chloramphenicol content.

Several preparations of β -glucuronidase were made by the methods described by Graham (10) and Fishman (11). One of the latter preparations contained 2.34 mg. of N per ml. by micro-Kjeldahl analysis, and showed an activity of 4000 units per ml. by using a phenolphthalein glucuronide substrate (12). 2 ml. of this enzyme preparation were added to 120 ml. of 0.1 N acetate buffer at pH 5.0 containing approximately 15 mg, of the chloramphenical glucuronic acid conjugate, and the mixture was incubated at 38°. At various time intervals aliquots of the solution were removed by pipette and heated in a boiling water bath for several minutes. This treatment was found to inactivate the enzyme without destroying chloramphenicol or hydrolyzing the glucuronide conjugate. The samples were filtered and analyzed for total nitro compounds by the titanium reduction method, and for unaltered chloramphenicol by the solvent extraction method and by microbiological assay. The results presented in Table II indicate that the antibiotic is reformed from the inactive conjugate. The hydrolysis appears to follow the kinetics of a first order reaction over the first 4 hour period. Under identical conditions with heat-inactivated enzyme added to the glucuronide, no antibiotic activity appeared.

Hydrolysates prepared in a similar manner were chromatographed on paper strips to demonstrate the presence of chloramphenicol and glucuronic acid. The nitro compounds were chromatographed in n-butanol containing 2.5 per cent phenol and 2.0 per cent pyridine, and colors were developed with the titanium-bromine diazotization reagents already described. The presence of glucuronic acid was established by chromatography with n-butanol containing 10 per cent acetic acid, and the bands were made visible by spraying with ammoniacal silver nitrate and heating, as described by Partridge (13). Controls were run with conjugate plus heat-inactivated enzyme, and with known preparations of chloramphenicol and sodium glucuronate added. It was found that bands corresponding to chloramphenicol and glucuronic acid appeared after enzymatic hydrolysis of the conjugate. A trace of reducing material was also observed in the enzyme preparation itself with an R_r of 0.30, but this did not interfere with the identification of glucuronic acid.

Isolation of Hydrolysis Products—The identification of chloramphenical and glucuronic acid as hydrolysis products was confirmed by direct isolation of chloramphenical, and by preparation of known derivatives of glucuronic acid.

A solution containing 800 mg. of chloramphenical glucuronide in 85 ml. of 0.12 m acetate buffer at pH 4.5, was incubated with 15 ml. of a β -glucuronidase preparation for 44 hours at 39°. Microbiological assay showed that 59 per cent of the theoretical antibiotic activity had appeared. The pH of the solution was adjusted to 6.5 and was then extracted four times with equal volumes of ethyl acetate. The extracts were pooled and

TABLE II

Hydrolysis of Chloramphenicol-Glucuronic Acid Conjugate with β -Glucuronidase. The glucuronide of chloramphenicol isolated from human urine was incubated with β -glucuronidase at pH 5.0 and 38°. All the results are expressed as micrograms of chloramphenicol equivalents per ml. of solution. The colorimetric method is used to determine total nitro compounds, while the solvent extraction and microbiological methods are used for active antibiotic compounds.

Time of incubation		Liberated		
This of Incubation	Colorimetric	Microbiological	Solvent extraction	chloramphenicol
min.	μgm. per ml.	μgm. per ml.	μgm. per ml.	per ceni
5	62.1	1.5	1.1	1.8
10	61.5	2.4	2.0	3.3
20	63.9	4.7	4.1	6.7
40	62.5	9.1	8.3	13.5
80	62.0	17.4	15.5	25.2
160	62.6	32.0	27.4	44.6
242	62.3	39.0	34.0	55.2
615	61.2	43.0	41.9	68.2
1440	63.5	45.0	45.9	74.8
Control	61.9	<1	<1	0

dried over anhydrous magnesium sulfate. The crystalline residue was dissolved in 5 ml. of hot water and recrystallized, yielding 177 mg. of crystalline material (72 per cent recovery). The compound melted at 149–150° and produced no depression of melting point when mixed with a known sample of chloramphenicol. The ultraviolet absorption and microbiological activity showed characteristics identical with those of chloramphenicol.

The aqueous residue from the preceding extraction was then evaporated under reduced pressure to 18 ml. and dialyzed in cellophane casing against 200 ml. of distilled water overnight in the refrigerator. The dialysate was evaporated to 10 ml. under reduced pressure at 50°, and the resulting

solution showed reducing activity by the Fishman modification of the Miller-Van Slyke procedure (11) close to the theoretical amount of glucuronic acid liberated by hydrolysis, and gave a positive color test for glucuronic acid by the naphthoresorcinol reaction.

An attempt was made to prepare a derivative of glucuronic acid which could be readily isolated and identified. The procedure used was similar to that described by Lohmar *et al.* (14), in which glucuronic acid is first oxidized to saccharic acid with bromine, and the insoluble dibenzimidazole derivative is then formed by condensation with *o*-phenylenediamine.

9 ml. of the dialysate concentrate described above were shaken with 1.45 gm. of barium benzoate and bromine at 0° for 2 hours, and then allowed to stand 70 hours at room temperature. The solution was then aerated to remove excess bromine, acidified with sulfuric acid, and the precipitate of benzoic acid removed by filtration. The filtrate was then treated with 0.33 gm. of silver carbonate in the dark for 2 hours, and the resulting solution was filtered, extracted three times with chloroform, and then evaporated to dryness. The residue was dissolved in acid and the solution treated with 280 mg. of o-phenylenediamine and 1 ml. of diethylene glycol at 135° for 2 hours. After decolorizing with norit, the pH was adjusted to 9 to 10 with ammonium hydroxide and the solution allowed to stand for 2 days. The solution was then acidified with HCl to pH 2 and extracted twice with ether and twice with ethyl acetate, following which it was made alkaline with ammonia and extracted three times with ether. The desired product was not extracted under these conditions. Upon seeding with less than 0.5 mg. of a known preparation of the dibenzimidazole of saccharic acid, the derivative crystallized.

The product was filtered off after 20 hours, yielding 58 mg. (m.p. 215–220°), with no depression of the melting point of the known dibenzimidazole (14). Upon recrystallization from ammoniacal solution, a product was obtained with a melting point of 225–230°. No depression of melting point was observed when mixed with a purified sample of saccharic acid dibenzimidazole. The known compound showed a melting point of 225–230° at a corresponding stage of purification, and 235° after recrystallizing.

Comparative ultraviolet absorption data for the benzimidazole derivatives of gluconic and saccharic acids, as well as for the compound described above, are presented in Table III. Three absorption maxima were observed at 245, 273.5, and 280 m μ . The absorption characteristics of the unknown were closely parallel to those of saccharic acid dibenzimidazole, but the $E_{1m}^{1\%}$ values were somewhat lower, due to the presence of impurities.

The recrystallized material was also converted to the picrate by treatment with picric acid in hot aqueous solution. After several recrystal-

lizations, the derivative was found to melt at 211-212°, with no depression of the melting point of the known picrate (14).

Degradation Studies with Periodic Acid—Theoretically glucuronic acid might be conjugated with chloramphenical through the —OH groups in either the 1 or 3 position in the side chain. After liberating the free amine by alkaline hydrolysis, oxidation with periodic acid would be expected to yield p-nitrobenzaldehyde from the 3-glucuronide, whereas formaldehyde would result from oxidation of the 1-glucuronide, as shown below:

$$O_2N$$
 $CHOH$
 CH
 CH_2OR
 CHO
 CHO
 CHO
 CHO
 CHO
 CH_2OR
 CHO
 The simultaneous degradation of glucuronic acid might also be expected, but it was found that this side reaction could be minimized by controlling temperature and reaction time. By using 0.1 n to 1.0 n NaOH at room temperature for 20 hours, the alkali-treated glucuronide was found to take up approximately 0.8 equivalent more periodate than the control in 15 minutes at 0°, which is to be expected if only the amide linkage is being hydrolyzed. Since the strength of alkali does not appear to affect the results, it may be assumed that the glucosidic linkage is stable within these limits. However, oxidation for periods greater than 15 minutes resulted in increased uptake of periodate with time, presumably due to cleavage of the glucuronic acid fragment. Similar behavior was observed with a preparation of menthol glucuronide.

200 mg. of chloramphenicol glucuronide were dissolved in 9 ml. of 0.1 n NaOH and kept at room temperature for 20 hours. The solution was then neutralized with 5 n sulfuric acid and cooled to 0°. A solution of 10 ml. of ice-cold periodate (containing 200 mg. of HIO₄·2H₂O and neutralized with bicarbonate) was added. After 15 minutes at 0° the reaction mixture was extracted four times with equal volumes of ether. The ether extracts were evaporated and the residue was recrystallized from 2 ml. of hot water, yielding 26.1 mg. of crystalline material. The product

was identified as p-nitrobenzaldehyde with a melting point of 104-106° and showed no depression of melting point when mixed with the known compound.

The aqueous residue was then treated with 0.5 ml. of 2 m arsenite and 0.4 ml. of 20 per cent KI. After 15 minutes, the pH was adjusted with acetic acid to 4 to 5 and 1.5 ml. of dimedon reagent were added (100 mg. per ml. of absolute ethanol). A precipitate began to form in 5 minutes and was filtered off after 3 hours. On recrystallizing from 1 ml. of 50 per cent methanol, 9.3 mg. of solids were obtained which melted at 186–188° and showed no depression of melting point when mixed with a known sample of formaldehyde dimedon.

Controls were run with the amino diol (II) to establish recovery figures for the oxidation products. With 85 to 90 per cent recovery of p-nitro-

Table III

Ultraviolet Absorption Characteristics of Hexuronic Acid Derivatives $E_{1\,\mathrm{cm.}}^{1\%}$ values shown in the body of the table were calculated for the three absorption maxima observed in aqueous solutions.

Compound	Ultraviolet absorption maxima, $E_{1 \text{ cm.}}^{1\%}$					
	$\lambda = 245 \text{ m}\mu$	$\lambda = 273.5 \text{ m}\mu$	$\lambda = 280 \text{ m}\mu$			
Gluconic acid monobenzimidazole	224	284	256			
Saccharic "dibenzimidazole	334	417	387			
Derivative prepared from chloramphenical conjugate	300	399	354			

benzaldehyde, the corrected yield from the glucuronide is 61 to 65 per cent. With a recovery of 70 per cent for the formaldehyde dimedon, the corrected yield is 14 to 18 per cent. The discrepancies in yield may perhaps be accounted for by the presence of impurities or by a slower rate of oxidation with periodic acid. However, it would appear that the principal form of the chloramphenicol-glucuronic acid conjugate is the 3-glucuronide, while the occurrence of a small amount of 1-glucuronide has not been ruled out.

We are indebted to many individuals for their kind assistance at various stages of this work. Urine specimens were obtained from hospitalized patients through the courtesy of Dr. George Chittenden and Dr. Elwood Sharp. Dr. Gertrude Rodney gave valuable assistance in the preparation of enzymes, and numerous assays were made by Miss Loretta Wolf and Mrs. Margaret Galbraith. The ultraviolet and infra-red absorption data

were obtained by Dr. John M. Vandenbelt, and the elementary analyses were performed by Mr. Charles Childs.

We are particularly indebted to Dr. A. Calvin Bratton, Jr., Dr. Harry M. Crooks, Jr., and to Mr. Leonard Doub for their helpful criticism and suggestions throughout the course of this work.

SUMMARY

Following the administration of chloramphenicol, the principal nitro compounds found in the urine of rat, dog, and man are (I) unchanged chloramphenicol, (II) a hydrolysis product of chloramphenicol, and (III) a conjugate of chloramphenicol with glucuronic acid. Of these, only chloramphenicol exhibits significant antibiotic activity.

Chloramphenicol and its conjugate with glucuronic acid have been isolated from human urine and identified. The glucuronide is readily hydrolyzed with the enzyme β -glucuronidase, yielding unchanged chloramphenicol and glucuronic acid. The hydrolysis products have been identified by paper partition chromatography, by direct isolation of chloramphenicol, and by the preparation of saccharic acid dibenzimidazole from glucuronic acid. The structure of this glucuronide is further established by degradation studies with periodic acid.

BIBLIOGRAPHY

- 1. Bartz, Q. R., J. Biol. Chem., 172, 445 (1948).
- Rebstock, M. C., Crooks, H. M., Jr., Controulis, J., and Bartz, Q. R., J. Am. Chem. Soc., 71, 2458 (1949).
- Glazko, A. J., Wolf, L. M., Dill, W. A., and Bratton, A. C., J. Pharm. Exp. Therap., 96, 445 (1949).
- Ley, H. L., Smadel, J. E., and Crocker, T. T., Proc. Soc. Exp. Biol. and Med., 68, 9 (1948).
- 5. Glazko, A. J., Wolf, L. M., and Dill, W. A., Arch. Biochem., 23, 411 (1949).
- 6. Joslyn, D. A., and Galbraith, M., J. Bact., 54, 26 (1947).
- 7. Consden, R., Gordon, A. H., and Martin, A. J. P., Biochem. J., 38, 224 (1944).
- Glazko, A. J., McGinty, D. A., Dill, W. A., Wilson, M. L., and Ward, C. S., J. Biol. Chem., 179, 409 (1949).
- 9. Craig, L. C., J. Biol. Chem., 155, 519 (1944).
- 10. Graham, A. F., Biochem. J., 40, 603 (1946).
- 11. Fishman, W. H., J. Biol. Chem., 127, 367 (1939).
- 12. Talalay, P., Fishman, W. H., and Huggins, C., J. Biol. Chem., 166, 757 (1946).
- 13. Partridge, S. M., Biochem. J., 42, 238 (1948).
- Lohmar, R., Dimler, R. J., Moore, S., and Link, K. P., J. Biol. Chem., 143, 551 (1942).

ERYTHROCYTE PYROPHOSPHATASE IN HEALTH AND DISEASE

II. REVERSIBLE AND IRREVERSIBLE INACTIVATIONS OF THE ENZYME

By B. NAGANNA*

(From the Department of Biochemistry, Andhra Medical College, Visakhapatnam, India)

(Received for publication, August 2, 1949)

It was previously reported (1) that erythrocyte pyrophosphatase is greatly inactivated by incubation with M/35 veronal-acetate buffer for 30 minutes prior to assay, and that the presence of magnesium ions affords a certain amount of protection from such inactivation. Jacobsen (2) reported that the inorganic pyrophosphatases of liver, kidney, and bone were completely destroyed by warming for 5 minutes at 55°. That inorganic pyrophosphatase is highly labile was contended by Pillai (3) working with muscle. In view of such contradictory findings on the nature of the enzyme from various tissues it was thought worth while to study the rapid inactivation of the erythrocyte enzyme in more detail.

Since the protective action of Mg ions on the enzyme against what was regarded as heat inactivation, and since the enzyme requires free SH groups for its action, it was assumed that the inactivation observed with buffer alone might be due to the oxidation of thiols, resulting in a decrease in the adsorptive capacity of the enzyme, which is thus rendered incapable of reacting with the magnesium or more probably with the magnesium pyrophosphate molecule. That magnesium protects other thiol enzymes from inhibitions produced by oxygen was indicated by Dickens (4) who suggests that magnesium forms a complex with the SH groups. Several workers (4-6) have found that oxygen inactivates thiol enzymes. In addition to the inactivation of the enzyme by incubation for $\frac{1}{2}$ hour with buffer, it was also found that the enzyme could be inactivated by bubbling oxygen gas slowly through the buffered enzyme solution for 15 minutes at 28°.

Considering the above two types of inactivation, one has to assume that the enzyme has essential groups, presumably thiols, which are so highly susceptible both to small changes of temperature and to oxygen that spontaneous inactivation might take place. But, on the other hand, it was found that, though the aqueous enzyme is gradually inactivated at room temperature, it retains more than 50 per cent of its original activity

^{*} Present address, Department of Chemistry, Veterinary College, Vepery, Madras 7, India.

even after 6 hours at 28°. Further, the undiluted hemolysate, *i.e.* 1:10 erythrocytes, when incubated at 38° for 15 minutes suffered only negligible inactivation (sometimes a slight activation too was observed).

Experiments were therefore repeated with water instead of buffer, to ascertain whether the buffer takes any part in the above inactivations, and it was found that in both cases the effect was slight when compared to that produced with buffer. It was thus recognized that the veronal-acetate buffer contributes to the major part of the inactivation. The results of the study of the inactivation by the buffer constituents and substances related to them, the reversibility of the inactivations, and protection against the inhibitors are detailed in this paper.

EXPERIMENTAL

Standard Assay—The procedure for the assay of the enzyme is the same as that given previously (1) except that the incubation period was reduced from 30 to 15 minutes.¹

Effect of Buffer on Enzyme—It is well known that not only the pH optimum of an enzyme varies, but also that the extent of enzyme action is specifically influenced by different buffers. Wisansky (7) observed that invertase action is depressed when the concentration of the neutral buffer acid (acetate) at the same optimum pH is increased. Hence the inhibitory action of the buffer acids on the pyrophosphatase might be anticipated.

The pyrophosphatase has the definite advantage in having an optimum pH near the neutral point, and it exerts an enzyme action at pH 7.0 which is as much as 75 to 80 per cent of that at the optimum pH. Thus in an unbuffered aqueous medium (pH 7.0) the activity is 82 per cent in one case and 77 per cent in another (Table I). Table I also shows the marked inhibitory effects of the buffer acids compared to the slight inhibition with water.

However, prolonged incubation with water also produces marked inactivation, as is shown in Table II. The pyrophosphate ion seems to be a more efficient protector than the magnesium ion.

Inhibition Experiments

In order to find out whether the inhibition was due to any specific effect of either of the buffer constituents, further experiments were set up. It

¹ Unless otherwise stated the incubation mixture has a final volume of 5 ml. at pH 7.6, with a final concentration of 0.02 m magnesium chloride and 0.001 m sodium pyrophosphate. Buffer quantities are varied as indicated in each case. 2.5 ml. of 10 per cent trichloroacetic acid were used for deproteinization. 5 ml. portions of the filtrate are always used for the estimation of the orthophosphate with a series of suitable standards. Blanks are set up simultaneously. As far as possible the experiments are run in duplicate. In many cases the duplicates agreed within 5 per cent, and whenever they differed by more than 10 per cent the experiments were repeated.

Table I Influence of Veronal-Acetate Buffer on Erythrocyte Pyrophosphatase

In Experiment 1 the enzyme is directly assayed by adding 0.5 ml. of the hemoly-sate (1:10 erythrocytes) to a mixture of 3 ml. of m/35 veronal-acetate buffer or water, 1 ml. of magnesium chloride (0.1 m), and 0.5 ml. of 0.01 m sodium pyrophosphate, brought to 38°. In Experiment 2, 0.5 ml. of the enzyme is incubated with 3 ml. of m/35 veronal-acetate buffer (pH 7.6) or 3 ml. of water (pH 7.0) for 15 minutes at 38°, and then treated with magnesium and pyrophosphate as in Experiment 1, and the enzyme assayed.

periment	Enzyme activity	Activity as per cent standard			
No.	Enzyme activity	Potent enzyme	Weak enzyme		
1	In buffer, pH 7.6 " water, " 7.0	100 82	100 77		
2	After incubation with buffer, pH 7.6, for 15 min. at 38° After incubation with water, pH 7.0, for	17	<10		
	15 min. at 38°	76	63		

TABLE II Protection of Enzyme by Pyrophosphate and Magnesium Ions

In Experiment 1, 0.5 ml. of the enzyme (1:10 erythrocytes) is treated with 3 ml. of m/35 veronal-acetate buffer, or with 3 ml. of buffer + 1 ml. of 0.1 m magnesium, or with 3 ml. of buffer + 0.5 ml. of 0.01 m pyrophosphate, and each incubated for 30 minutes at 38°. In Experiment 2, 5 ml. of the enzyme are treated with 10 ml. of magnesium, or 5 ml. of pyrophosphate + 5 ml. of water, or 10 ml. of water, and each incubated for 3 hours. The enzyme activity was measured in each case with 1.5 ml. portions after adding the requisite quantities of magnesium or pyrophosphate and bringing the volume to 5 ml. with buffer (2 ml.) and water. Assay period, 15 minutes; temperature, 38°; pH 7.6.

Experiment No.	Enzyme incubated with	Activity as per cent standard assay
1	Buffer alone	11
	" + pyrophosphate	51
	" + magnesium	32
2	Pyrophosphate	42
	Magnesium	29
	Water	<10

was found that acetate as well as veronal sodium was inhibitory. Not only the buffer constituents but substances related to them also produced inhibition.²

² Regarding the molecular constitution and the accessibility to the enzyme, reference might be made to the exhaustive study of Quastel and Wooldridge (8) on dehydrogenating bacterial enzymes, and the work of Murray (9) on lipase.

From Figs. 1 to 3, it can be seen that luminal is more inhibitory than veronal. The inhibitory effect is more marked with weaker enzymes (Fig. 2), with which incubation with water alone produces about 20 per cent inhibition in 30 minutes. Further, it will be noted that the extent of inhibition with narcotics, as well as with malonate and acetate, increases with the length of incubation. Similar results were reported (10) on the action of narcotics on brain dehydrogenation.

Since malonic acid and urea form the two parts of the barbiturate structure, and since malonic acid is found to be the more inhibitory part (Fig. 3), it might be concluded that inactivation by narcotics was due to the

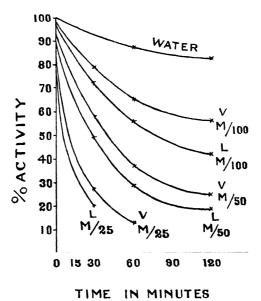


Fig. 1. Action of veronal and luminal. 0.5 ml. of the enzyme was treated with 3 ml. of water or the reagents of varying strengths at pH 7.0, and incubated at 38° for varying periods of time, and then assayed by adding 0.5 ml. of 0.01 m sodium pyrophosphate and 1 ml. of 0.1 m magnesium chloride. L, luminal sodium; V, veronal sodium.

malonyl radical. No doubt higher concentrations of urea produced inhibition, but with such high concentrations even Mg ions produced comparatively more effect. Since acetate is not so markedly inhibitory as the malonate, it might be that the carboxyl group exerts the inhibitor effect. The study was therefore extended to other carboxylic acids, the results of which are presented in Table III. The extent of the inactivation varied somewhat with the same inhibitor on different enzyme preparations, the weaker preparation becoming inactivated sooner. Hence, for purposes of

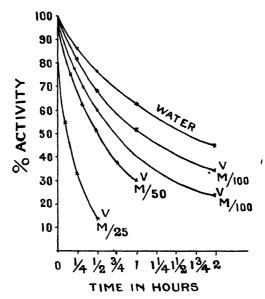


Fig. 2. Action of veronal. The experimental procedure is the same as is given below Fig. 1, but a weaker enzyme preparation was employed.

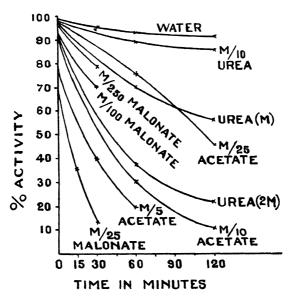


Fig. 3. Action of acetate, malonate, and urea. The experimental procedure is the same as given for Figs. 1 and 2, with a strong enzyme preparation.

comparison, the data obtained with different enzyme preparations of nearly the same potency are given in Table III.

Of the aldehydes formaldehyde is the most toxic. This inhibitory effect

Table III
Inhibition of Erythrocyte Pyrophosphatase with Various Substances

05 ml of the enzyme (1:10 erythrocytes) was incubated with 3 ml of the inhibitor and adjusted to pH 7 0 for different time periods, as shown, at 38° Controls incubated with water set up simultaneously. After the incubation the enzyme is treated with the requisite quantities of magnesium and pyrophosphate and assayed in unbuffered aqueous medium. Final volume, 5 ml, period of hydrolysis, 15 minutes, temperature, 38°, pH 7 0. The per cent activity of the control and of the others was calculated, from which the per cent inhibition was noted by difference in each

Inhibitor		Per cent Inhibition Preliminary incubation period			Per cent inhibition Preliminary incubation period	
				Inhibitor		
		15 min	30 min		15 min	30 min
02 м sod	ium acetate	60	80	01 м sodium glutamate		70
01"	"	45	64	01" " citrate	90	90
01"	" formate	43		01" " adipate		71
01"	" propionate	40	65	01" " benzoate	İ	68
01"	" butyrate	45	65	01 " glucose		5
01"	" chloroacetate	47		01" phenylalanine	8	
01"	'' iodoacetate	90	90	01 "tryptophan		80
01 " ace	tamide	8	14	0 01 м "	1	15
01 " gly	cine	6	11	0 01 "tyrosine		72
01 " alaı	nine	4		0 005 m cystine	Ì	82
01 " sod	ıum pyruvate		90	01 m creatine	1	32
01"	' lactate	ļ	72	01 " creatinine		8
01"	' mandelate		76	01 "guanidine		90
01"	' oxalate	90	90	01 " thiourea		90
01"	' malonate	90	90	01 " urethane	į	31
01"	' succinate	30	48	01 " formaldehyde	90	
	' aspartate	28	51	01 " acetaldehyde		84
01"	' fumarate	90	90	01 "benzaldehyde		36
01"	' maleate	90	90	01 " semicarbazide		51
01"	' malate		90	01 "sodium diethyl di-		
01"	' tartrate		90	thiocarbamate		81
01"	' glutarate		75			

cannot be due to the reactivity of the carbonyl group alone, since in the carboxylic acids the carbonyl group is not so reactive as that in aldehydes or ketones. Almost the same result is observed with all the fatty acids tested as well as with benzoic acid. These results suggest that the car-

boxyl group might be responsible for the inhibition. Replacement of the —OH in the —COOH with —NH₂ seems to decrease the inhibitor effect markedly (note acetamide in Table III). Also an electronegative group adjacent to the carboxyl seems to increase and an electropositive group like —NH₂ seems to annul the inhibitor effect. Thus glycine is non-inhibitory, and sometimes with lesser concentrations and shorter incubation periods it actually produced slight activation. In this connection it will be interesting to refer to the findings of Bodansky (11) who observed that glycine in higher concentrations inhibits bone and intestinal phosphatases and that the introduction of methyl groups into the amino group nullifies the inhibitor effect of the amino acid.

Malonate, oxalate, and citrate inhibition must be due to their greater number of carboxyl groups, but curiously enough, the dicarboxylate, succinate, is not even so inhibitory as acetate. However, when a double bond or hydroxyl groups are introduced, as in fumaric and maleic acids and malic and tartaric acids, there is greater inhibition than with succinate, while with the introduction of an amino group, as in aspartate, there is neither increase nor decrease in the inhibitor effect compared to succinate. Perhaps on account of steric effects only one carboxyl in succinate might be effective, whereas both the carboxyls might be effective in fumaric, maleic, malic, and tartaric acids. The finding (Table VI) that fumaric and malic acids are more inhibitory than maleic and tartaric acids, respectively, can be explained as due to steric hindrance. Similar steric effects due to different isomers of tartaric acids on acid phosphatase were noted by Abul-Fadl and King (12).

It will be observed that with propionate, pyruvate, lactate, and mandelate the hydroxyl group increases the inhibitor effect of the acid, and the keto group adjacent to a carboxyl group seems to impart more toxic properties. Similar conclusions were drawn by Dixon (13) regarding the types of lachrimators which inhibit thiol enzymes.

Creatine shows comparatively little inhibition, which is not the case with creatinine,³ indicating that a free carboxyl group is probably necessary for inhibition, but such an assumption cannot be valid in the case of ascorbic and dehydroascorbic acids (not shown in Table III) in which the —COOH is not free. Further, substances containing hydroxyl groups cannot be inhibitive unless —COOH is also present. Thus glucose is practically non-inhibitory.

Acetone is not so inhibitory as acetaldehyde. Acetone, like urea, inhibits only in molar concentrations. Substitution of the —S— or —NH— group for the —O— of urea bestows marked inhibitory properties. Thus

³ In the case of creatinine the trichloroacetic acid filtrates should be diluted suitably before the phosphate is estimated.

thiourea and guanidine are as strongly inhibitory as is malonic acid. Semi-carbazide and diethyl dithiocarbamate caused inhibitions, while urethane produced only a slight effect. Both ethyl and methyl alcohols are inhibitory, but as the experiments were done under different conditions, the data are not shown.

Protection and Reactivation Experiments

To obtain more information on the nature of the reactions it was considered desirable to conduct experiments on reversal of the inhibitions. First of all the possible activators were tested.

It has already been reported (1) that bile salt is an activator. Cysteine and glutathione (GSH) were found to be good activators. Sodium sulfide and hyposulfite showed activation in some experiments, but unfortunately the sulfide especially also produced inhibition in some cases, particularly when the enzyme was kept in contact with them for longer periods. Their effects were not investigated further, and the data are therefore not presented. Cysteine is the most potent activator of all the substances tested. Different results are reported on the action of thiol compounds on various phosphatases (14-19), which are generally regarded as inhibitory. Roche et al. (20) found that intestinal pyrophosphatase (pH 7.8) is inhibited by cysteine, the inhibition disappearing in the presence of iodoacetic acid. They have also reported (21) that cysteine, GSH, Fe, Mn, and Zn produce a slight activation of liver pyrophosphatase (pH 7.4), and that the cysteine and Fe couple cause marked activation. The effect of thiols on the erythrocyte pyrophosphatase does not seem to have been investigated by them, though they found (22) activation of erythrocyte acid phosphatase by cysteine as well as by GSH.

It is found that cysteine is a strong activator of erythrocyte pyrophosphatase, and that the addition of Fe does not increase cysteine activation. Cysteine activation with fresh enzyme is not so marked as it is with old enzyme. Preliminary incubation with the activator for 15 minutes is necessary for maximum activation. Omission of this preliminary incubation sometimes produced negligible activation with fresh enzymes. Incubation at room temperature is preferable, since in a few cases the results were not so satisfactory when the incubation was carried out at 38°.

Vitamin C—Vitamin C is reported as an activator of certain phosphatases by some workers and as an inhibitor by others, while King and Delory (23) proved definitely that vitamin C has no action on serum alkaline phosphatase. Roche and others (20) have reported inhibition of intestinal pyrophosphatase (pH 7.8) with vitamin C, and working with mammalian erythrocytes (22), found a strong activation of acid pyrophosphatases (pH 3.8 and 5.8) with vitamin C. Maschmann and Helmert and Purr (24, 25)

reported that papain is inhibited by vitamin C alone, but is activated by the vitamin C-Fe complex. It has been found that vitamin C is fairly inhibitory to erythrocyte pyrophosphatase (Table IV). The inhibition was high except in a few samples. At no time was any activation observed with either vitamin C or vitamin C-Fe complex. The latter is definitely inhibitory. The most interesting finding is that dehydroascorbic acid also is an inhibitor, and is more toxic than ascorbic acid in many cases. Thus the effect of ascorbic acid has no relation to its oxidation-reduction poten-

Table IV Effect of Vitamin C and Cysteine on Enzyme

50 ml. of the hemolysate (1:10 human erythrocytes) were shaken with 5 ml. of chloroform and divided into two equal portions, of which one was kept in the refrigerator and the other at room temperature (28°). The next day, 0.5 ml. portions of both were treated with 0.5 ml. aliquots of the reagents, and, after 15 minutes standing at room temperature, the activities were determined by adding 2.5 ml. of buffer (pH 7.6), 1 ml. of 0.1 m magnesium chloride, and 0.5 ml. of 0.01 m pyrophosphate. The reagents, cysteine, and vitamin C were freshly prepared and aliquots added to the enzyme immediately after the reaction was adjusted to pH 7.6 with NaOH. Assay period, 15 minutes; temperature, 38°; pH 7.6.

					Enzyme k	ept at 4°	Enzyme kept at 28°		
					Per cent activation (+) or inhibition (-)	Per cent of original activity	Per cent activation (+) or inhibition (-)	Per cent of original activity	
Enzym	e alone.					98		13	
ü	+ 1.58	mg.	cysteine		+110	206	+136	31	
"	+ 3.15	"	"		+116	212	+255	46	
"	+6.30	"	"		+126	222	+318	55	
"	+9.50	"	"		+126	222	+373	62	
"	+1.76	"	vitamin	C	-65	34	-100	0	
"	+ 3.52	"	"	"	-86	12	-100	0	
"	+ 5.28		"	"	-98	0	-100	Ō	

tial, and the inhibition must have been brought about by virtue of its structural configuration.

Tables IV and V illustrate the results obtained with cysteine, cysteine-Fe complex, vitamin C, and vitamin C-Fe complex. Blanks were run for each experiment in these cases.

Protection Experiments

Table VI gives the results of protection of the enzyme with pyrophosphate and magnesium. 0.5 ml. of the enzyme is treated with 0.5 ml. of water, pyrophosphate, or magnesium, and then 0.5 ml. of the inhibitor (pH 7.6) is added to each, the mixture incubated at 38° for 30 minutes, and then the

activity measured. A control without the inhibitor was also set up to assess the extent of inactivation produced by incubation with water alone. Two weak enzymes, E_1 and E_2 , are employed for marked inhibition. With stronger inhibitors a relatively potent enzyme, E_3 , is used.

The results in Table VI show that both pyrophosphate and magnesium are able to protect the enzyme almost completely except in the case of iodoacetic acid and guanidine. Even spontaneous inactivation due to water is prevented. The fact that the concentration of the pyrophosphate is a tenth that of the inhibitor suggests that the pyrophosphate is adsorbed at the active centers and thus blocks the inhibitors.

Table V

Effect of Vitamin C-Fe⁺⁺ and Cysteine-Fe⁺⁺ Complexes on Enzyme

An old enzyme, which has lost most of its activity, was used. 0.5 ml. portions of the enzyme were treated with 0.5 ml. of the reagents containing varying amounts of freshly prepared and neutralized solutions of cysteine, vitamin C, and ferrous ammonium sulfate. After 15 minutes standing at room temperature, the activities were measured as in Table IV.

	Reagents added	Per cent activation (+) or inhibition (-)
Enzyn	ne alone	
"	+ 7.9 mg. cysteine·HCl	+165
"	+ 7.9 " $+ 0.39$ mg. Fe ⁺⁺	+117
46	+7.9 " +0.19 " "	+139
"	+ 1.58 " $+ 0.39$ " "	+41
"	+ 1.58 " + 0.19 " "	+61
"	+ 0.39 " Fe ⁺⁺	+13
"	+ 0.39 " + 2 mg. vitamin C	-100
* *	+0.19 " " +2 " " "	-80
"	+ 2 mg. vitamin C	-100

Since the concentration of Mg ions is greater than that of the inhibitor, it can be argued that the protection might be due only to combination of Mg with the inactivator, leaving no inhibitor to react with the enzyme. Such a possibility was recently discussed in another connection by Pardee and Potter (26). But in the present study one would expect, in such an instance, that the enzyme inactivated by incubation with the inhibitor should be capable of being reactivated with Mg. And since no such reversal could be achieved, it is feasible to assume that Mg and pyrophosphate have strong affinities for the active centers and thus are rapidly adsorbed; and, since the inhibitors act only gradually, they fail to produce inactivation when the enzyme is already saturated with Mg or pyrophosphate. Both pyrophosphate and Mg fail to protect the enzyme against certain inhibitors like copper, fluoride, guanidine, etc.

TABLE VI Protection of Enzyme

0.5 ml. of the enzyme was treated with 0.5 ml. of water or 0.01 m pyrophosphate or 0.1 m magnesium, and then with 0.5 ml. of the inhibitor adjusted to pH 7.6. A control with 0.5 ml. of water was also set up in each case. All were incubated at 38° for 30 minutes and the remaining activities estimated by adding the requisite quantities of magnesium and pyrophosphate as in the standard assay, the volume of buffer being adjusted to make the final volume to 5 ml. Different enzymes (E_1 , E_2 , E_3) are used. Assay period, 15 minutes; temperature, 38°; pH 7.6.

	Per cent activity remaining after treatment								
Inhibitor	w	ater-trea		Pyrophosphate-treated enzyme			Magnesium-treated enzyme		
	E ₁	E2	E ₁	Eı	E2	E.	E ₁	E2	E,
Water	74	61	94	101	83	100	100	79	101
0.1 m formaldehyde		!	<10			48			<10
0.1 " formate	49	46		97	81		98	79	1
0.1 " acetate	41	35		98	87		96	85	
0.1 " chloroacetate	37	31	İ	100	93		99	89	
0.1 "iodoacetate	6	0	21	35	28	69	17	13	52
0.1 " malonate	17	10	25	100	98	98	88	81	90
0.1 " oxalate		1	<10			92			71
0.1 " succinate		l	54	i	i	104			102
0.1 "aspartate		•	51		l	100			98
0.1 " malate			33	ļ	}	98			99
0.1 " tartrate			53	Ì	ł	102			101
0.1 " fumarate		1	15	1		96			97
0.1 " maleate			26	ļ		99			98
0.1 " citrate			<10			97			96
0.1 "guanidine			18			34			23
0.1 " thiourea			26	1		100			91
0.01 m ascorbate			37			100			93
0.01 " dehydroascor-					}				l
bate*			21	1		95			78
0.01 m alloxan			64	}	1	92			68
0.001 m copper sulfate.			<10	İ	1	<10			<10
0.001 " sodium fluor-					1				į
ide			<10			19			14

^{*} Dehydroascorbic acid is prepared by treating ascorbic acid solution with norit.

Reversal Experiments

A few inhibitors were selected to ascertain the extent the activators cysteine and bile salt could reverse the reactions. The enzyme is incubated with the inhibitors for 15 minutes and then with cysteine or bile salt for another 15 minutes and the activities are measured. For purposes of comparison, incubations were carried out at room temperature (28°) and at 38°. The results are given in Table VII.

TABLE VII Reversal of Inhibition by Cysteine and Bile Salt

0.5 ml. of the enzyme was incubated with 0.5 ml. of the reagents for 15 minutes, and the activities were then measured, as shown in Column A. Two sets of similarly treated and incubated enzymes were treated with 0.5 ml. of 0.5 per cent sodium taurocholate (Column B) or 0.5 ml. of 0.1 m cysteine (Column C), and incubated for another 15 minutes and the activity measured. The preliminary incubation of Enzyme E_1 was carried out at room temperature, and of Enzyme E_2 at 38° . Enzymes E_3 and E_4 were incubated with the inhibitors at 38° and with the activators at 28° . All of the reagents were adjusted to pH 7.6. Assay period 15 minutes; temperature 38° ; pH 7.6.

Reagent added	Per cent activity			Reagent added	Per cent activity		
Enzyme E ₁				Enzyme E ₂			
	(A), 28°	(B), 28°	(C), 28°		(A), 38°	(B), 38°	(C), 38°
Water 0.001 M copper sul-	93	130	135	Water	97	92	88
fate 0.001 m sodium fluo-	<5	<5	87	0.1 m oxalate	<5	<5	60
ride	< 5	11	17	0.1 " malonate	12	22	37
0.1 m formaldehyde	<10	< 5	<10	0.1 "citrate	<5	< 5	55
0.1 "iodoacetate	27	26	31	0.1 " maleate	47	65	37
0.1 " acctate	59	103	78	0.1 " thiourea	18	15	75
0.01 m alloxan	64	97	109	0.1 " guanidine	<5	< 5	60
0.04 " veronal so-				5 mg. ascorbic acid	18	20	84
dium	61		99				
Enzyme E₄				Enzyme E ₄			
	(A), 38°	(B), 28°			(A), 38°		(C), 28°
Water	100	105		Water	100		105
0.1 м acetate	70	90		0.1 м acetate	76		85
0.05 м veronal sodium	41	40		0.05 м veronal so-			
0.05 " alloxan	<5	< 5		dium	41		61
0.05 " oxalate	< 5	< 5		0.05 " alloxan	<5		52
0.05 " malonate	62	76		0.05 " oxalate	19		59
0.05 " citrate	<5	< 5		0.1 "malonate	24		43
0.1 м fumarate	26	54		0.1 "fumarate	31		27
0.1 " maleate	54	72		0.1 " maleate	61		53

DISCUSSION

It is difficult to understand the mechanism by which fluoride inhibition is brought about, because pyrophosphate and magnesium could not protect the enzyme nor could the bile salt and cysteine reverse the fluoride inhibition. Copper inactivation must be due to its action on the enzyme thiols

since cysteine reverses the inhibition. The negligible protection by Mg and partial protection by pyrophosphate against formaldehyde, whose inhibition is irreversible either with bile salt or with cysteine, suggest that the inhibition must have been brought about by interaction with the amino groups of the enzyme, and that Mg protects only the SH groups and not the amino groups, whereas pyrophosphate could protect the amino groups also. More or less similar results are obtained with iodoacetate. iodoacetate produce inhibition by reacting also with amino groups? a possibility was first suggested by Michaelis and Schubert (27) and later by others (28, 29). The results of Williams and Watson (19) and Bodansky (31) indicate the possibility of amino groups inhibiting alkaline phosphatases, which could be reversed by iodoacetate. Though it is generally believed that iodoacetate is a specific thiol inhibitor (30), it might not be correct to regard all enzymes that are inactivated by iodoacetate as thiol enzymes, or vice versa (32). Hence it is more likely that iodoacetate inhibition of the enzyme is brought about primarily by its action on amino groups.

Alloxan inhibition could be reversed partly with bile salt and completely with cysteine, provided the concentration of the alloxan was low and the incubations were performed at room temperature. Higher concentrations and incubation at 38° produced inhibitions which could not be reversed with bile salt and could only be partly reversed with cysteine. Acetate inhibition is reversed to a greater extent with bile salt than with cysteine, indicating some kind of blocking or shielding of the thiols by acetate.

Veronal inhibition could not be reversed with bile salt, but reversal occurs to a certain extent with cysteine. Malonate inhibition could be reversed only to a small extent either with cysteine or with bile salt. Oxalate and citrate are more toxic than malonate, the inhibitions being irreversible with bile salt. But their inhibitions, as well as those due to entirely different compounds like thiourea, guanidine, and ascorbic acid, could be reversed to a large extent with cysteine.

Results with fumarate and maleate offer additional difficulties in correlating the above findings. Morgan and Friedmann (33) claim that maleic acid is a specific thiol inhibitor, whereas fumaric acid has no action on thiol enzymes. Further, different thiol enzymes are inhibited to a varying extent with maleic acid (34). In the present study it was found that fumarate is more toxic than maleate and that cysteine could not reverse their inhibitions. The fumarate inhibition has only to be explained as due to shielding the active centers by the trans isomer, the steric effects due to the cis isomer being only slight. Reversal with bile salt supports such an assumption. How far maleate reacts with the thiols producing some fumarate simultaneously and how far this rearranged isomer contributes to the

inhibition it is difficult to assess, but such probabilities exist. In passing it will be interesting to note the findings of Greenberg and Winnick (35).

The results with guanidine and thiourea are interesting and need to be studied more extensively. Both seem to react with the thiols, since cysteine reverses their inhibitions. But they differ from each other in that the pyrophosphate and Mg could protect the enzyme only against thiourea and not against guanidine. Work on the effect of these two reagents on other thiol enzymes might perhaps throw some light on the nature of their action.

Besides the thiol groups indicated by the copper and alloxan inhibitions and reversal with cysteine, and the amino groups indicated by the irreversible inhibitions with formaldehyde and iodoacetic acid, there is no indication of any probable presence of a metallic group as an active group. Though diethyl dithiocarbamate produced inhibition in higher concentrations, the reagent in smaller concentration as well as sodium azide did not cause inhibition, but, on the other hand, sometimes produced slight activation owing probably to the removal of trace metals present as impurities.

Until precise quantitative data on the kinetics of the inhibitions detailed in this paper are available, any conclusions drawn are only tentative. Since obtaining the enzyme in a purer form is prerequisite, work is being carried out in this direction.

SUMMARY

- 1. The rapid inactivation of erythrocyte pyrophosphatase on incubation with veronal-acetate buffer was found to be due to the inhibiting action of both the buffer constituents, viz. veronal and acetate.
- 2. Several monocarboxylic and dicarboxylic acids were studied and it was found that all inhibit the enzyme. The inhibitory action of the carboxyl group of the acetate is increased by substituting another carboxyl group in the acetate as in malonate, whereas introduction of an amino group as in glycine abolishes the original inhibitory action of the carboxyl. The intact carboxyl group seems to exert an inhibitory effect, since acetamide is practically non-inhibitory.
- 3. Though more carboxyl groups increase the inhibitor effect, the spatial configuration of the molecule decides the extent of inhibition. The proximity of the carboxyls seems to enhance the inhibitor effect. Oxalate is a very strong inhibitor, malonate is less inhibitory, and succinate is not as inhibitory as acetate. However, fumarate and maleate are more inhibitory than succinate, and curiously enough the trans isomer is as strongly inhibitory as malonate, while maleate is much less inhibitory.
- 4. Urea is non-inhibitory, whereas thiourea and guanidine are strongly inhibitory.

- 5. Both ascorbic and dehydroascorbic acids are inhibitory.
- 6. Among the amino acids tested, glycine, alanine, and phenylalanine are non-inhibitory, whereas tyrosine, tryptophan, and cystine are inhibitory.
- 7. Both pyrophosphate and magnesium ions are found to protect the enzyme to a large extent against many of the inhibitors. Against copper and fluoride they failed to protect the enzyme. Magnesium could not protect the enzyme against formaldehyde inhibition, while 50 per cent protection is obtained with pyrophosphate. Both showed poor protection against guanidine. Pyrophosphate had relatively more effect than magnesium against oxalate, iodoacetate, alloxan, and dehydroascorbic acid.
- 8. Besides sodium taurocholate, cysteine and glutathione are found to be activators of the enzyme. Sodium hyposulfite and hydrogen sulfide also produced some activation, but in some experiments they caused inhibition also. Cysteine is found to be the most potent activator, while cystine is an inhibitor.
- 9. Fe alone is very slightly activating, but addition of Fe neither reverses vitantin C inhibition, as in the case of papain (24, 25), nor does it increase cysteine activation, as in the case of liver pyrophosphatase (21).
- 10. Inactivation due to copper, alloxan, dicarboxylates except maleate and fumarate, ascorbate, veronal, acetate, guanidine, and thiourea could be reversed to a variable extent with cysteine. Bile salt could reverse to a small extent the inhibition due to fumarate and maleate, malonate and acetate. Neither cysteine nor bile salt could reverse the inhibition due to formaldehyde and iodoacetate. Fluoride inhibition could be reversed only to a negligible extent by both.
- 11. The various aspects of the inhibition, protection, activation, and reversal of the inhibitions have been discussed, and it is suggested that free sulfhydryl and the amino groups are essential for the enzyme action.

I am grateful to Dr. D. Narayana Rao who gave me facilities and encouragement throughout this investigation, and to Dr. N. K. Iswaran for his help in securing the experimental material. My thanks are due to Dr. O. Bodansky, Dr. E. J. King, Dr. J. Roche, Dr. A. Sols, Dr. V. N. Patwardhan, and Dr. T. R. Seshadri for their kindness in supplying materials, and to Mr. Theophyles Samuel and Mr. H. C. Friedmann for translating the French and German literature cited in this work. My special thanks are due to Dr. G. Gopalarao for his valuable discussions.

BIBLIOGRAPHY

- 1. Naganna, B., and Narayanamenon, V. K., J. Biol. Chem., 174, 501 (1948).
- 2. Jacobsen, E., Biochem. Z., 263, 302 (1933).
- 3. Pillai, R. K., Biochem. J., 32, 1087 (1938).

- 4. Dickens, F., Brochem. J., 40, 145 (1946).
- 5. Mann, P. J. G., and Quastel, J. H., Biochem. J., 40, 139 (1946).
- 6. Haugaard, N., J. Biol. Chem., 164, 265 (1946).
- 7. Wisansky, W. A., J. Am. Chem. Soc., 61, 3330 (1939).
- 8. Quastel, J. H., and Wooldridge, W. R., Biochem. J., 22, 689 (1928).
- 9. Murray, D. R. P., Biochem. J., 23, 292 (1929).
- 10. Davies, D. R., and Quastel, J. H., Biochem. J., 26, 1672 (1932).
- 11. Bodansky, O., J. Biol. Chem., 165, 605 (1946).
- 12. Abul-Fadl, M. A. M., and King, E. J., Biochem. J., 42, p. xxviii (1948).
- 13. Dixon, M., Biochem. J., 42, p. xxvi (1948).
- Waldschmidt-Leitz, E., Schaffner, A., and Scharikova, A., Z. physiol. Chem., 214, 75 (1933).
- 15. Lohmann, K., Biochem. Z., 262, 157 (1933).
- 16. Bersin, Z. physiol. Chem., 222, 177 (1933).
- 17. Schaffner, A., and Bauer, E., Z. physiol. Chem., 225, 245 (1934).
- 18. Kutscher, W., and Worner, A., Z. physiol. Chem., 239, 109 (1936).
- 19. Williams, H. L., and Watson, E. M., J. Biol. Chem., 135, 337 (1940).
- Roche, J., Laromiguiriere, S., and Laurens, A., Bull. Soc. chim. biol., 25, 1019
 (1943).
- 21. Roche, J., Thoai, N., and Milhan, O., Bull. Soc. chim. biol., 25, 1217 (1943).
- 22. Roche, J., Thoai, N., and Bandoiu, J., Bull. Soc. chim. biol., 24, 1247 (1942).
- 23. King, E. J., and Delory, D. E., Biochem. J., 32, 1157 (1938).
- 24. Maschmann, E., and Helmert, E., Z. physiol. Chem., 223, 127 (1934); 224, 56 (1934).
- 25. Purr, A., Biochem. J., 29, 13 (1935).
- 26. Pardee, A. B., and Potter, V. R., J. Biol. Chem., 178, 241 (1949).
- 27. Michaelis, L., and Schubert, M. P., J. Biol. Chem., 106, 331 (1934).
- 28. Weinstein, S. S., and Wynne, A. M., J. Biol. Chem., 112, 649 (1935-36).
- 29. Purr, A., Biochem. J., 29, 5 (1935).
- 30. Dickens, F., Biochem. J., 27, 1141 (1933).
- 31. Bodansky, O., J. Biol. Chem., 174, 465 (1948).
- 32. Weil, L., and Kocholaty, W., Biochem. J., 31, 1255 (1937).
- 33. Morgan, E. J., and Friedmann, E., Biochem. J., 32, 733, 862 (1938).
- 34. Peters, R. A., and Wakelin, R. W., Biochem. J., 40, 513 (1946).
- 35. Greenberg, D. M., and Winnick, T., J. Biol. Chem., 135, 761 (1940).

UTILIZATION OF OPTICAL ISOMERS OF METHIONINE AND FORMYLMETHIONINE BY SOME LACTOBACILLI

By JOSEPH R. SPIES AND DORRIS C. CHAMBERS

(From the Allergen Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, October 29, 1949)

Reports on the utilization of the optical isomers of methionine by lactobacilli are conflicting. Stokes and Gunness (1) reported that Lactobacillus arabinosus does not utilize D-methionine. Dunn et al. (2) reported that D-methionine was inactive in producing growth of L. arabinosus and Leuconostoc mesenteroides, and Lyman et al. (3) made a similar observation with L. mesenteroides and Streptococcus faecalis R. However, Henderson and Snell (4) stated that D-methionine has partial activity with S. faecalis R in the presence of L-methionine, and Steele et al. (5) claimed that D-methionine was 10 per cent as active as the L isomer with L. mesenteroides.

The object of this investigation was to determine the relative degrees of utilization of pure optical isomers of methionine, formylmethionine, and racemic DL-methionine by L. arabinosus, L. mesenteroides, and S. faecalis R.

EXPERIMENTAL

DL-Methionine was resolved by a previously described (6) modification of the methods of Windus and Marvel (7) and Jackson and Block (8), in which purity of the isomers rather than high yields was stressed. Analytical data and optical rotations showing the purity of the isomers obtained by resolution of two 30 gm. quantities of formyl-dl-methionine are recorded elsewhere (6). The dl-methionine used for comparison with l-methionine was obtained by recrystallization of 2 gm. of analytically pure dl-methionine from 75 ml. of 67 per cent ethanol. The product weighed 1.4 gm. and contained 9.45 per cent nitrogen; theoretical, 9.39 per cent.

Cultures of *L. arabinosus* 17-5, *L. mesenteroides* P-60, and *S. faecalis* R were obtained from the American Type Culture Collection, 2029 M Street, Washington, D. C.

Microbiological analyses were made with the medium of Henderson and Snell (4) except that pyridoxine was used instead of pyridoxal; the volume of medium used was 1 ml. and that of the sample was 1 ml. Organisms were allowed to grow for 66 to 72 hours in the dark at 30°. The samples were then diluted to 50 ml., and the lactic acid was titrated with 0.05 N

sodium hydroxide, brom thymol blue being used as indicator. Conventional standard curves were obtained from the average results of duplicate or quadruplicate tests over the concentration range of 2, 4, 6, 8, and 10 γ of L-methionine (Run II (6)) per test.

RESULTS AND DISCUSSION

It is apparent that D-methionine in a DL mixture is not utilized by L. arabinosus and L. mesenteroides because DL-methionine was only 49 ± 1 per cent and 51 ± 1 per cent as active with these organisms, respectively, as was L-methionine. These results were determined from a standard curve

Table I
Growth Responses of Lactobacilli to D-Methionine and Formyl-D- and L-Methionine

		Amount of Growth*		
Substance	Quantity used per test	L. arabinosus	L. mes- enteroides	S. faecalis R
	γ	per ceni	per cent	per cent
D-Methionine	12	3.5	1.8	0.0
	20	3.8	2.4	0.0
Formyl-p-methionine	12	2.2	0.0	0.7
	20	2.8	0.0	1.4
Formyl-L-methionine	12	3.1	2.2	t
	20	4.7	3.1	l †

^{*} Approximately maximum growth of S. faecalis R, L. mesenteroides, and L. arabinosus was obtained with 12, 12, and 16 γ of L-methionine, respectively. The amount of growth produced by each substance was estimated by determining the apparent weight of L-methionine in the given weight of each substance tested, multiplying by 100, and dividing by the above approximate quantities of L-methionine, which gave maximum growth with respective organisms.

made by averaging the values of quadruplicate tests at concentration levels of 0, 2, 4, 6, 8, and 10 γ of L-methionine and from the average of quadruplicate tests containing 4, 8, and 12 γ of DL-methionine. D-Methionine in a DL-mixture appeared to have partial activity with S. faecalis R as noted by Henderson and Snell (4), but the amount of activity was not determined because results of various tests were erratic.

The activities of p-methionine alone and of formyl-p- and L-methionine are shown in Table I. None of the organisms was capable of utilizing appreciable amounts of p-methionine or formyl-p-methionine when 12 and 20 γ of each were used per test. Formyl-L-methionine was not utilized by L. arabinosus or L. mesenteroides, but it was utilized by S. faecalis R as well or slightly better than was free L-methionine. The growth of S.

[†] Maximum growth.

faecalis R was 12 ± 4 per cent greater with the formyl derivative than with L-methionine as determined by the average of quadruplicate tests with quantities of formyl-L-methionine equivalent to 2, 4, and 8 γ of L-methionine. Simmonds and Fruton (9-11) recently reported that a prolineless mutant of Escherichia coli grew better in a medium containing L-proline peptides than in one containing equimolar quantities of proline. A possibly similar relationship of utilization mechanisms between S. faecalis R and formyl-L-methionine and L-methionine is recognized.

These observations are pertinent to the concept that microorganisms require free amino acids for growth. Thus S. faecalis R is not able to utilize benzoyl-detryptophan as shown by Stokes et al. (12) or acetyl-detryptophan (13). Although it is not known whether this organism can utilize formyl-detryptophan, it is apparent that utilization of N-acyl amino acids by lactobacilli is not determined generally by the nature of the acyl group because L. arabinosus and L. mesenteroides cannot utilize formyl-determine. This observation indicates need for further investigation to determine the fundamental relationship of amino acid derivatives to their utilization by various microorganisms.

SUMMARY

Lactobacillus arabinosus and Leuconostoc mesenteroides do not utilize p-methionine alone or in the presence of L-methionine in pl-mixtures. Streptococcus faecalis R does not utilize p-methionine alone, but in the presence of L-methionine it appears to make partial use of the p form. None of these organisms utilized formyl-p-methionine. L. arabinosus and L. mesenteroides did not utilize formyl-L-methionine, but S. faecalis R gave 12 per cent more growth with this derivative than with free L-methionine. Need for further study of the relationship of amino acid derivatives to their utilization by microorganisms is pointed out.

Addendum—Since this paper was submitted for publication, Camien and Dunn (14) have shown that L arabinosus 17-5 utilizes p-methionine and primethionine as well as does rimethionine in a synthetic medium containing at least 1 γ per cent of pyridoxamine or 10 γ per cent of pyridoxal. Pyridoxine is, however, ineffective in promoting utilization of p-methionine with this organism. The ineffectiveness of pyridoxine in promoting utilization of p-methionine by L arabinosus is confirmed by the present work.

BIBLIOGRAPHY

- 1. Stokes, J. L., and Gunness, M., J. Biol. Chem., 154, 715 (1944).
- Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., J. Biol. Chem., 163, 577 (1946).
- Lyman, C. M., Moseley, O., Butler, B., Wood, S., and Hale, F., J. Biol. Chem., 166, 161 (1946).

- 4. Henderson, L. M., and Snell, E. E., J. Biol. Chem., 172, 15 (1948).
- Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., J. Biol. Chem., 177, 533 (1949).
- 6. Spies, J. R., J. Biol. Chem., 182, 439 (1950).
- 7. Windus, W., and Marvel, C. S., J. Am. Chem. Soc., 53, 3490 (1931).
- 8. Jackson, R. W., and Block, R. J., J. Biol. Chem., 122, 425 (1937-38).
- 9. Simmonds, S., and Fruton, J. S., J. Biol. Chem., 174, 705 (1948).
- 10. Simmonds, S., and Fruton, J. S., Science, 109, 561 (1949).
- 11. Simmonds, S., and Fruton, J. S., J. Biol. Chem., 180, 635 (1949).
- Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., J. Biol. Chem., 160, 35 (1945).
- 13. Spies, J. R., and Chambers, D. C., Anal. Chem., 21, 1249-1266 (1949).
- 14. Camien, M. N., and Dunn, M. S., J. Biol. Chem., 182, 119 (1950).

MAGNESIUM VINYL PHEOPORPHYRIN a₅, ANOTHER INTERMEDIATE IN THE BIOLOGICAL SYNTHESIS OF CHLOROPHYLL

By S. GRANICK

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, October 27, 1949)

Among the mutants arising after irradiation of *Chlorella vulgaris* with x-rays, three yellow mutants have been found, the absorption spectrum of whose ether extracts contained bands at 623 and 572 m μ . These bands were suggestive of protochlorophyll or a related compound. However, the concentration of this pigment was so low that its isolation in a crystalline state was not feasible. The pale green compound has therefore been characterized principally by the solubility properties and quantitative absorption curves of a number of its derivatives. On the basis of these properties the compound has been identified as Mg vinyl pheoporphyrin a_5 , *i.e.* protochlorophyll, lacking phytol (Fig. 10).

The molar extinction coefficients of a number of the pertinent compounds, measured in dioxane, are presented in Table I, where E mole = $\log_{10} (I_0/I) \times (1/(\text{cm.} \times \text{mole per liter}))$.

EXPERIMENTAL

General Characteristics of Mutant—One of these mutants, i.e. strain 31, contained somewhat more of the pale green pigment than did the two others and has been used for identification of the pigment. The three mutants have similar characteristics. When grown in the dark on a solid medium of agar, glucose, and inorganic salts (1), they form deep yellow colonies tinged faintly greenish. They were found to contain carotenoids and a trace of chlorophyll besides the two-banded pale green pigment. When grown in the light, the cells become deep green and the two bands at 623 and 572 mu are no longer detectable. In the light these mutants behave like wild type Chlorella, growing in the absence of glucose, i.e. photosynthesizing; when such green cultures are transferred to the dark, they maintain their green color for weeks, as does the wild type. Inocula taken from mutants grown in the light and seeded on a glucose medium give rise again in the dark to deep yellow colonies. In wild type Chlorella enzymes are present which bring about the production of chlorophyll in the dark as well as in the light. In contrast to the wild type, this yellow Chlorella mutant resembles the higher plants in requiring light for the production of chlorophyll.

Growth on solid medium containing glucose was best at 20-25°, a maximum yield being obtained in 7 to 9 days. For example the relative yield of packed cells at 20-25° was 1.3 cc.; at 15° it was 0.5 cc.; at 28-34° it was 1.1 cc.; and in the light on the same medium it was 1.5 cc. The yield of the pale green pigment was not appreciably affected by changing the conditions of growth or by enrichment of the medium with various substrates and vitamins.

TABLE I

Molar Extinction in Dioxane × 10⁻⁴

Wave-lengths in $m\mu$ (upper figures of the pairs). The extinction (lower set of figures) was read with the Beckman spectrophotometer.

	Maximum I	Maximum II	Maximum III	Maximum IV	Soret
Posi	ition of	maxima			
Vinyl pheoporphyrin as mono-	638	588	567	525	419
methyl ester	0.215	1.40	1.770	0.825	19.3
Pheoporphyrin a _b monomethyl	634	583	562	521	417
ester	0.188	1.261	1.606	0.939	19.3
Pheoporphyrin as dimethyl ester	625	573	55 0	512	
oxime	0.194	1.074	1.440	1.290	
Chloroporphyrin e, trimethyl ester	629	576	543	506	
	0.170	0.673	0.809	1.263	
Vinyl chloroporphyrin es trimethyl	633	579	550	512	409
ester	0.130	0.720	1.04	1.14	20.5
Po	sition of	minima			
Vinyl pheoporphyrin as mono-	623	575	543	475	
methyl ester	0.110	1.15	0.490	0.180	
Pheoporphyrin as momethyl ester	619.5	571. 5	541		
	0.087	0.928	0.516		
Pheoporphyrin as dimethyl ester	610	562	533		
oxime	0.077	0.520	0.470		
Vinyl chloroporphyrin e6 trimethyl	620	5 65	530	465	
ester	0.085	0.480	0.410	0.201	

For the study of the pigment the cells were grown in the dark in Blake flasks. Each flask containing 700 cc. of medium (agar-glucose-inorganic salts) was inoculated with cells obtained from one agar test-tube slant. After 6 to 7 days at room temperature the cells were harvested by washing with about 20 cc. of water per flask and then centrifuging. The supernatant was tinged pale green and overlay a thin layer of young pale green cells and cellulose wall débris. The main bulk of the cells below this

layer was deep yellow in color, consisting of large cells filled with starch granules. The total volume of packed cells per flask was about 5.0 cc.

Pigments of Chlorella Mutant—The harvest of one flask was treated with 40 cc. of acetone and after 1 hour was centrifuged. The residue was then extracted with 40 cc. of methanol and finally with 20 cc. of ether. The extracts were combined, diluted with water, and the ether layer was washed with water. A few cc. of saturated aqueous NaCl aided in the separation of the layers. The washed ether solution was diluted to 50 cc. with ether and the absorption spectrum of the total pigment extract was measured with a Beckman spectrophotometer in cells of 1 cm. light path (Curve A, Fig. 1).

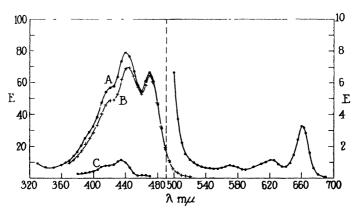


Fig. 1. Relative extinction in ether of pigments of Chlorella mutant 31 per flask of medium. Curve A represents extinction of total pigments. Curve B is the total absorption of the carotenoids. Curve C, obtained by difference, represents the extinction of the green pigments in the ultraviolet; the extinction of the green pigments in the visible region is represented by Curve A in the visible region.

To determine the absorption of the green pigments in the ultraviolet region it is necessary to remove the carotenoid pigments. The green pigments are made water-soluble with methyl alcoholic KOH and the absorption of the yellow pigments is measured (Curve B); the difference between the extinction values of Curve B and Curve A is plotted in Curve C, which is the absorption spectrum of the green pigments in the ultraviolet region. The separation of the green and the yellow pigments was brought about by adding to a small separatory funnel 2.5 cc. of the ether extract plus 2.5 cc. of petroleum ether (b.p. 60–70°) plus 3 cc. of 30 per cent KOH in absolute methanol. The mixture was shaken intermittently for 5 minutes. Then 3 cc. of water and 3 cc. of ether were added. The upper yellow ether layer was removed. A trace of yellow pigment re-

mained in the lower layer, to which was added 1 cc. of methanol; the solution was then extracted with 5 cc. of ether. The yellow ether extracts were combined, washed with water, and diluted to 25 cc. This volume corresponds to a 1:10 dilution of the original stock ether solution. No fluorescence with ultraviolet light was detectable in this yellow ether solution, indicating the absence of porphyrin or dihydroporphyrin pigments. The absorption of this extract is presented in Curve B of Fig. 1.

The band maxima at 620 and 430 m μ are interpreted as representing Mg vinyl pheoporphyrin a_5 , and the maximum at 419 as that of vinyl pheoporphyrin a_5 . From the extinction maxima one may estimate that the 5 cc. of packed cells contained a total of about 0.16 mg. of chlorophyll a and approximately 2 mg. of yellow pigments.

Isolation and Absorption Spectrum of Pale Green Pigment-To obtain sufficient pigment the cells of mutant 31 were grown in batches of twenty to thirty Blake flasks in the dark on the agar-glucose-inorganic salt medium at 20-25°. They were harvested after 5 to 6 days, at a time when growth was not quite maximum, in order to diminish the yield of certain decomposition products. The cells and supernatant were stirred with 50 mg. of Na₂CO₃ and then treated with 4 volumes of acetone. After centrifugation the aqueous acetone extract was transferred to a separatory funnel and extracted three times with petroleum ether (b.p. 50-60°) to remove chlorophyll, carotenoids, and fatty substances. No absorption band at 625 mu was present in the petroleum ether solution, indicating the absence of protochlorophyll. The acetone extract was diluted with water, acidified with M/15 KH₂PO₄, and extracted into ethyl ether twice and once again after addition of aqueous saturated NaCl. The resulting pale green ether solution was washed with water and then extracted with 0.05 m ammonia buffer at pH 9.5. It was then returned to ether and concentrated to a volume of 25 cc. The total yield of pigment in this extract was about 0.5 mg.

The absorption spectrum of the ether solution is shown in Fig. 2, Curve A. The position of the band maxima at 623, 570, 530, and 432 m μ corresponds to the position of the band maxima of protochlorophyll (2), but the ratios of the extinction maxima of the bands do not correspond to those of protochlorophyll. On analysis it was found that this absorption spectrum may be accounted for by assuming a mixture of Mg vinyl pheoporphyrin a_5 and vinyl pheoporphyrin a_5 . Since the extinction values of Mg vinyl pheoporphyrin may be assumed when corrected by a weight factor to be identical with its phytyl ester (i.e. protochlorophyll), the extinction values of Koski and Smith (2) have been used, together with the extinction values of synthetic vinyl pheoporphyrin. From the extinction values at 623 and 419 m μ the experimental Curve A was found

to be 1.2×10^{-5} M with respect to vinyl pheoporphyrin and 2.10×10^{-5} M with respect to the Mg complex. By subtracting the extinction values of vinyl pheoporphyrin from Curve A, the points on Curve B were obtained. Curve B should correspond to the absorption spectrum of pure Mg vinyl pheoporphyrin a_5 . For protochlorophyll minus its phytol the ratios of E (the molar extinction coefficient) for the band maxima at 623, 571, and 432 m μ are 1.0:0.427:9.30. For Curve B the ratios are 1.0:0.440:9.07. The agreement is satisfactory and indicates that the absorption of the pale green extract from *Chlorella* was due primarily to vinyl pheoporphyrin and its Mg complex.

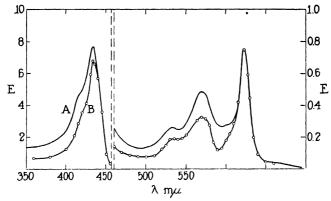


Fig. 2. Relative extinction of the pale green pigment isolated by extraction, indicating that the solution contains a mixture of vinyl pheoporphyrin a_5 and its Mg complex. Curve A represents total pigment. Curve B represents the magnesium complex. The points on Curve B were obtained by subtraction of extinction values of vinyl pheoporphyrin; the ratios of the maxima of Curve B agree with published data on the Mg complex.

Quantitative Determination of Mg^{++} in Complex—On shaking some of the pale green ether solution with 1 N HCl and washing out the acid, the ether solution was found to contain a porphyrin spectrum of the rhodo type (3). Evidently a metal atom was split out of the complex. Conditions were chosen to remove the metal, estimate it quantitatively, and at the same time recover the porphyrin for further study.

The pale green ether solution was washed with M/15 NH₄H₂PO₄ and then with water. The extinction value for this solution at 623 m μ was determined. Assuming it to be Mg vinyl pheoporphyrin a_5 , the solution was calculated to be 4.5 \times 10⁻⁶ M. Of this solution 170 cc. were concentrated to a volume of a few cc. and transferred with ether washings to a special graduated centrifuge tube whose lower 5 cm. portion had a capacity of 1 cc. and whose upper portion was flared out. To the deep green

ether solution of 3.5 cc. was added 0.1 cc. of 3 N HCl, and the acid was stirred into the ether by bubbling air through it with a capillary pipette. The acid was then neutralized exactly with 0.1 cc. of 3 N NaOH. The ether was removed by evaporation. Water was added to dilute the solution in the tube to 0.8 cc. and the tube was centrifuged to spin down the insoluble porphyrin. The resulting solution had a very faintly yellowish tinge, owing to a trace of colloidal porphyrin. Of this solution 0.1 cc. was used for the Mg determination by the Titan yellow method (see below). To correct for the absorption due to the colloidality, 0.1 cc. of the metal-containing solution was diluted to 1 cc. with the reagents except Titan yellow and its extinction was determined against a blank of the reagents without Titan vellow. The resulting extinction was subtracted from the colorimetric Mg reading. On the assumption of Mg vinyl pheoporphyrin and the extinction at 623 mu, the total ether sample used was calculated to contain 18.6 γ of Mg⁺⁺; found by the colorimetric procedure, 19.2 γ of Mg⁺⁺.

Spectrophotometric Comparison of Porphyrin with Vinyl Pheoporphyrin a_{δ} —The quantitative absorption spectrum of vinyl pheoporphyrin a_{δ} has not been reported in the literature. We prepared this compound from dried spinach leaves by way of chlorophyll a+b precipitation in petroleum ether (4), hydrolysis of the phytol with strong acid (5), separation and crystallization of pheophorbide a from ether (5), reduction of the dihydroporphyrin with iron and formic acid, its reoxidation by air to vinyl pheoporphyrin a_{δ} monomethyl ester, and its crystallization from ether (6). Calculated for $C_{3\delta}H_{34}O_{\delta}N_4$, C 71.2, H 5.76 per cent; found, C 70.42, H 6.15, and no ash.

The molar extinction in dioxane of this synthetic vinyl pheoporphyrin a_{δ} monomethyl ester (Fig. 3, I) is presented as the smooth curve, B, in Fig. 4. (Because of the great tendency toward peroxide formation of dioxane, this solvent was distilled over sodium and stored in small bottles filled to the brim, tightly stoppered, in the ice box.) The extinctions are identical for dioxane and ether as solvents, except that in ether the maxima are displaced about 1 m μ to the ultraviolet side.

The ether solution containing the pale green compound isolated from Chlorella was treated with 0.1 n HCl to split out the Mg⁺⁺. The ether solution was then washed with water and extracted successively with increasing concentrations of HCl. The main component, extractable between 2.7 and 3.3 n HCl (HCl No. \simeq 11), when transferred into ether had a four-banded rhodo type spectrum in the visible region whose most intense band was at 566 m μ .

Small amounts of other rhodo type substances were observed. One having an HCl No. \simeq 10 had its most intense band at 562 m μ ; another

Fig. 3. Derivatives prepared from Mg vinyl pheoporphyrin a_5 . Only the pyrrole Ring III and its adjacent atoms are shown.

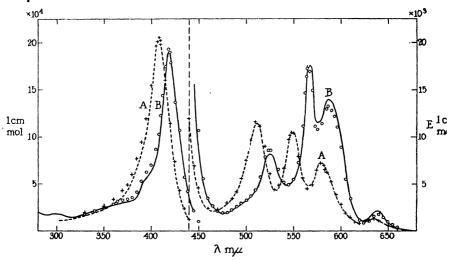


Fig. 4. Molar extinctions in dioxane of synthetic vinyl pheoporphyrin a_b (Curve B). Synthetic vinyl chloroporphyrin e_b (Curve A). The points on both curves represent extinction of compounds derived from the pale green pigment of *Chlorella* mutant 31.

having an HCl No. \simeq 7 had its most intense band at 558 m μ . A trace of a chlorophorpyrin-like spectrum with an HCl No. \simeq 0.4 was also observed once. These substances were noted to be increased especially when the cells were grown during the summer, the room temperature being

30-32°. The properties of these pigments suggested that they are decomposition products of vinyl pheoporphyrin, the main component.

To free the main component of the slight impurities the ether solution was extracted several times with 3.0 n HCl and the fraction was selected which was extractable between 3.0 and 3.3 n HCl. This acid solution was transferred to peroxide-free ether, the ether solution washed with water and evaporated, the residue dissolved in dioxane, and the absorption spectrum measured. The extinction at one point, i.e. 419 m μ , was multiplied by a factor to bring it to the molar extinction value of synthetic vinyl pheoporphyrin a_5 at this wave-length. All other points were multiplied by this same factor and are plotted as points on Curve B of Fig. 4. These points lie sufficiently close to the curve to suggest that the substance derived from the *Chlorella* mutant is vinyl pheoporphyrin a_5 . The deviation of the points from the curve in the band regions at 525 and 587 m μ is beyond experimental error, indicating traces of impurity, possibly vinyl phylloerythrine or vinyl 10-hydroxypheoporphyrin or both.

The solubility properties of this compound derived from *Chlorella* are also in agreement with those of vinyl pheoporphyrin a_5 monoester in the value of the HCl number and in the extractibility in aqueous ammoniacal solution from ether, which indicates an ionizable carboxyl group. No evidence for a phytol ester was obtained.

The general characteristics of the absorption spectrum also support the idea that the derivative from Chlorella is a pheoporphyrin. The rhodo type spectrum (the third visible band being strongest) is produced by a formyl, acetyl, or carboxyl group attached to a β -carbon of a pyrrole in the porphyrin. The presence of a C=O group in pheoporphyrin is signalized by a rhodo type spectrum. However, as compared to rhodoporphyrin (in which a —COOH is attached to a β -carbon) the spectrum in pheoporphyrin is shifted some 17 m μ to longer wave-lengths owing to the C=O being part of a cyclopentanone ring. The displaced rhodo type spectrum is given by porphyrin compounds which possess a five-or six-membered ring containing a C=O group attached at the β -carbon atom.

Vinyl Chloroporphyrin e_6 Trimethyl Ester (Fig. 3, II)—A characteristic reaction of pheoporphyrin with intact isocyclic ring (i.e. 10-carbomethoxy-0- γ -ethanone) is methanolysis, resulting in the splitting of this ring and the formation of a trimethyl ester derivative. This derivative is readily recognized by its characteristic four-banded etio or step-ladder type of absorption spectrum in the visible region. Phylloerythrine (Fig. 5, VII) does not undergo this reaction. 10-Hydroxypheoporphyrin a_5 (IV) is converted to chloroporphyrin a_7 trimethyl ester (VI) which possesses an etio type of spectrum.

The quantitative absorption spectrum of vinyl chloroporphyrin e_6 has not been reported in the literature. The trimethyl ester of this compound was prepared by adding to 6 mg. of crystalline synthetic vinyl pheoporphyrin a_6 15 cc. of a solution of dry methanol containing 30 per cent by weight of dry HCl. The solution was left overnight tightly stoppered at room temperature, then diluted with ice water, neutralized with sodium acetate, extracted into ether, and the ether solution fractionated with successively increasing concentrations of HCl. Two substances were present. The main component had an HCl No. $\simeq 2.2$, identifiable with the chloroporphyrin compound. It was crystallized from a mixture of chloroform and methanol. Its absorption spectrum in dioxane is represented

Fig. 5. Other products arising from vinyl pheoporphyrin. Only pyrrole Ring III and its adjacent atoms are shown.

as Curve A of Fig. 4. The other component, present in traces and of a rhodo type, had an HCl No. $\simeq 13$ to 14 and was presumably vinyl phylloerythrine methyl ester produced during the reaction.

The pheoporphyrin compound derived from *Chlorella* was also treated in the same way with methyl alcoholic HCl. The main fraction with HCl No. $\simeq 2.2$ was isolated, neutralized, dissolved in dioxane, and its absorption spectrum measured, the data being plotted as points on Curve A of Fig. 4. The points are in essential agreement with the curve of the synthetic compound.

When Chlorella strain 31 was grown at 30–32°, compounds suggestive of decomposition of pheoporphyrin were observed. The ether solution extracted with 2.5 to 4 n HCl contained a mixture of compounds of the rhodo type. This mixture when treated with methyl alcoholic HCl and

fractionated showed the presence of a relatively high content of a rhodo type compound with an HCl No. \simeq 10 to 14, presumably vinyl phylloerythrine methyl ester. A fraction with HCl No. \simeq 4 and the etio type of spectrum was presumably vinyl pheoporphyrin a_7 trimethyl ester, and a fraction with an HCl No. \simeq 2.5 contained vinyl chloroporphyrin e_6 triester with a trace of the vinyl pheoporphyrin a_7 triester.

Identification of Pheoporphyrin from Chlorella Strain 31 As Monovinyl Compound—The hypothesis was presented in previous papers that protoporphyrin (containing two vinyl groups) is a precursor of chlorophyll a (containing one vinyl group). At some intermediate stage one of the vinyl groups is reduced to an ethyl group. Absorption spectrum data indicate that the wave-lengths of a porphyrin band system are decreased some 4 m μ on reduction of one vinyl group and about 8 m μ on reduction of two vinyl groups (3). With this criterion it could be demonstrated that the pheoporphyrin a_5 isolated from Chlorella was a monovinyl pheoporphyrin.

2 mg. of synthetic vinyl pheoporphyrin a₅ dissolved in dioxane were added to 2 cc. of acetic acid containing 2.5 mg. of colloidal Pd, heated on a boiling water bath, and H₂ passed into the solution for 10 minutes. The green solution was reduced to a yellow-brown color with an absorption band at 490 to 500 mu. Water was then added and the solution transferred to a separatory funnel containing ether and shaken, and the compound permitted to autoxidize for 10 minutes. The fraction extractable between 2.5 and 3 N HCl was used. The resulting pheoporphyrin a₅ was dissolved in dioxane and its absorption determined. The extinction at 415 mu was multiplied by a factor to convert it to the molar extinction value at this wave-length as determined by Stern and Wenderlein (7) and all the other points were multiplied by this factor. The maxima and minima of the resulting smooth curve (Fig. 6) are in good agreement with data reported by Stern and Wenderlein on pheoporphyrin a₅. The fraction isolated from the Chlorella mutant and extractable with 3.0 to 3.3 n HCl from other solution was reduced in the way described above and isolated. Its absorption spectrum measured in dioxane, when converted by a factor to the coefficient of molar extinction, is represented as points on the smooth curve of Fig. 6. The wave-length maxima are decreased about 4 mu, providing supporting evidence that the original compound from Chlorella contained only one vinyl group.

As a further check on the identity of the Chlorella compound, the compound reduced at the vinyl group was heated in aqueous pyridine solution with hydroxylamine and Na₂CO₃ for 30 minutes at 100° and transferred to ether. A fraction was isolated by extraction of the ether solution with 1.5 N HCl. The absorption spectrum of the compound in dioxane

(Fig. 7), when multiplied by a factor to convert it to the coefficient of molar extinction, was compared with the data of synthetic pheoporphyrin oxime dimethyl ester (Fig. 3, III) as published by Stern and Wenderlein

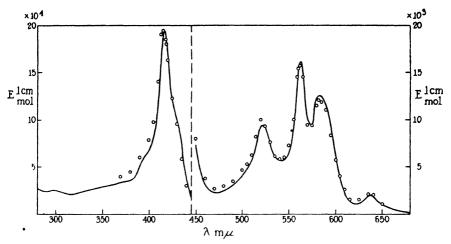


Fig. 6. Molar extinction of pheoporphyrin a_b in dioxane. The smooth curve represents the synthetic preparation. The points on the curve represent the compound derived from *Chlorella*.

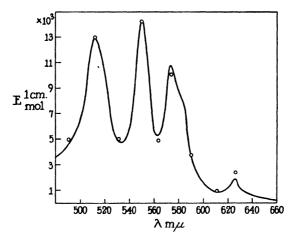


Fig. 7. Molar extinction of pheoporphyrin a_b oxime in dioxane. The points on the curve are those of the compound derived from *Chlorella*.

(8). The points of maxima and minima fit their published curve and furnish additional support for the identification of the compound isolated from *Chlorella*.

Solubility Properties of Protochlorophyll from Etiolated Barley—The absence of a phytol group in the vinyl pheoporphyrin a_5 compound isolated from the Chlorella mutant led us to scrutinize the evidence that protochlorophyll was a phytol ester. Noack and Kiessling (9), in their important contributions to protochlorophyll structure, isolated "protochlorophyll" from cucurbit seed coats and also from etiolated oat seedlings. The properties of this compound, namely too low a C content of the impure preparation, extraction from ether with HCl of 12 to 18 per cent concentration, solubility properties that did not change on treatment with phytol esterase (chlorophyllase), all were indicative of the absence of phytol. These authors concluded that they had obtained no direct evidence for the presence of phytol. Although Fischer and Oestreicher (10) eventually synthesized the phytol ester of vinyl pheoporphyrin a_5 , they did not determine the HCl number of their compound nor compare the solubility properties with protochlorophyll isolated from the plant.

The only direct evidence that protochlorophyll is a phytol ester is the Mg determination by Koski and Smith (2) of the protochlorophyll isolated from etiolated barley by chromatography, which agrees with the assumption of a phytol. Unfortunately no carbon analysis of this compound was made nor were significant solubility properties reported.

In order to obtain confirmatory evidence to support the presence of the phytol group we have studied the solubility of protochlorophyll from etiolated barley. In one experiment the etiolated seedlings were grown in six flats as described by Koski and Smith (2), harvested in the dark, and heated for 5 minutes in water at 90° to destroy the esterases. After the excess moisture was squeezed out, the material was ground with about 9 volumes of acetone in a blendor. The pigments were then extracted into petroleum ether (60-70°, b.p.) with the aid of saturated NaCl. Extraction of the petroleum ether solution with dilute aqueous 0.01 N NH₄OH removed about 10 to 20 per cent of the Mg pheoporphyrin compound, indicating that this was not esterified with phytol. To the main bulk of the Mg pheoporphyrin compound in petroleum ether were added 20 cc. of acetic acid and the petroleum ether was removed by evaporation at low pressure. The acetic acid solution was mixed with ethyl ether. and the ether solution was washed with water and was fractionated with increasingly higher concentrations of HCl in the cold room. A trace of vinyl pheoporphyrin a_5 was extracted with 4 N HCl, but the bulk of the compound was extractable with 8 n HCl; i.e., at an acidity somewhat less than was required to extract the traces of pheophorbide a phytyl ester. The hydrolysis of the ester in strong acid is no greater in protochlorophyll than in chlorophyll. These solubility properties indicate that protochlorophyll as it exists in the etiolated barley contains an alcohol highly insoluble in water in ester linkage; i.e., presumably a phytyl ester.

The absorption spectrum of this acid-extracted phytyl ester, transferred to ether, washed, evaporated, and dissolved in dioxane, was determined and multiplied by a factor to convert it to the coefficient of molar extinction. The points so obtained fall mainly on the smooth curve (Fig. 8) for synthetic vinyl pheoporphyrin a_5 . A slight impurity is suggested by the band at 585 m μ and a trace of pheophorbide is made evident by the band at 660 m μ .

In another experiment etiolated barley seedlings were ground in acetone, the final concentration of acetone being 60 to 70 per cent. The proto-

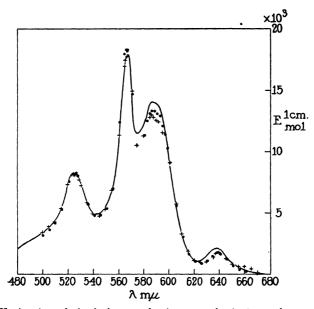


Fig. 8. Extinction of vinyl pheoporphyrin a_b synthetic (smooth curve). \times , compound isolated from etiolated barley seedlings heated in boiling water and then extracted with ether and acid; \bullet , compound isolated from etiolated barley without heating and extracted with ether and acid.

chlorophyll in the acetone extract, placed overnight in the ice box, lost its phytol. It became extractable from petroleum ether into a dilute alkaline aqueous solution, and was extractable from ether with 4 n HCl. The compound was crystallized from ether-methanol and its quantitative absorption spectrum in dioxane adjusted to the coefficient of molar extinction is plotted in Fig. 8.

These solubility properties may be considered as confirmatory evidence for the presence of a phytyl ester in the protochlorophyll molecule.

Some Experiments with Light—The lack of phytyl in the Mg vinyl pheoporphyrin compound obtained from the Chlorella mutant and the

production of chlorophyll in this mutant in the light indicate complex relationships. To study chlorophyll formation three Blake flasks were seeded and grown in the dark for 7 days and then exposed to 150 footcandles of fluorescent "daylight" at 6.5°. Quantitative extraction of the pigments was made as described above. The bands of Mg vinyl pheoporphyrin, originally present, disappeared and chlorophyll was formed in an amount equivalent to over twice this compound. At the same time there was a significant decrease in carotenoid absorption as compared to non-irradiated controls. In another experiment, flasks, similarly grown in the dark, were exposed to the light at a temperature of $+1^{\circ}$ to -2°

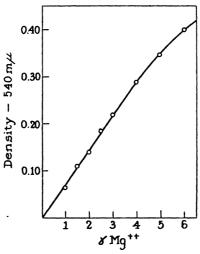


Fig. 9. Curve for microcolorimetric determination of Mg^{++} . The extinction values at 540 m μ of Mg in Titan yellow complex are plotted against Mg^{++} concentration.

for periods up to 12 hours. A progressive decrease in the Mg vinyl pheoporphyrin bands was observed with time without significant change in chlorophyll. A decrease in carotenoid absorption was also noted here.

Smith (11) demonstrated that protochlorophyll in etiolated barley was quantitatively converted to chlorophyll on illumination in the cold. The failure to observe chlorophyll formation at 0° in mutant 31 may be due to lack of phytol in the molecule, the Mg vinyl pheoporphyrin being destroyed on illumination. On illumination at 6.5° chlorophyll was synthesized, indicating that a chain of reactions including pheoporphyrin synthesis and esterification with phytol could occur at this temperature. Among other effects there seems to be an incomplete block to phytol esterification in this mutant.

Microcolorimetric Determination of Mg^{++} —The Titan yellow method of Ludwig and Johnson (12) was modified for the determination of microgram quantities of Mg^{++} . The colored solution with a total volume of 1 cc. is measured in a Beckman spectrophotometer at 540 m μ , with a narrow cell of 1 cc. capacity and a light path of 1 cm. (13). The extinction is measured against a blank containing all the reagents except Mg. The calibration curve for extinction against Mg concentration is a straight line up to about 4 γ of Mg (Fig. 9). The extinction values are stable for over an hour. The method is accurate to ± 4 per cent. Accurate pipetting of the reagents, especially of the Titan yellow, is essential. The reagents are added in the following order: Mg^{++} (as $MgSO_4 \cdot 7H_2O$ for standard), 1 N H_2SO_4 0.02 cc., H_2O to make the solution up to 0.5 cc. The following are then added: 0.10 cc. of 1 per cent soluble starch (recently boiled), 0.20 cc. of saturated aqueous solution of CaSO₄, 0.10 cc. of 0.05 per cent aqueous solution of Titan yellow, 0.10 cc. of 1 N NaOH.

DISCUSSION

The properties of the pale green pigment, isolated from the *Chlorella* mutant 31 have been shown to agree in all respects with those of Mg vinyl pheoporphyrin a_5 . The present status of the problem of biological chlorophyll synthesis may be represented by the scheme presented in Fig. 10.

By tracer techniques it has been shown that glycine and acetic acid or compounds derived from acetic acid are important building blocks in the formation of protoporphyrin (14). None of the intermediate steps to protoporphyrin is known. From Chlorella mutant W₅B protoporphyrin IX has been isolated. Magnesium porphyrin has been demonstrated in Chlorella mutant 60 (15). In this paper, evidence for the presence of Mg vinyl pheoporphyrin a_b has been presented. For transformation to this stage from Mg protoporphyrin it is necessary to postulate several intermediate steps; one is the reduction of one of the vinvl side chains to an ethyl side chain; other steps are the oxidation of the propionic acid side chain, the esterification of this side chain with methyl alcohol, and the cyclization to form the cyclopentanone ring. The next step is the esterification of the second propionic acid group of Mg vinyl pheoporphyrin to form protochlorophyll. The last step, the reduction of protochlorophyll to chlorophyll, occurs in wild type Chlorella as an enzymic process in the dark. In Chlorella mutant 31 and in higher plants this process takes place in the light.

Two interesting relationships may be noted here. The two prominent pigments of protoplasm, namely heme and chlorophyll, are related structurally since they are derived from the same precursor, protoporphyrin IX.

Fig. 10. Scheme of biosynthesis of chlorophyll

These two pigments are also related functionally as expressed in the following equation.

$$O_2 + 4H^+ + 4(e) = \frac{\text{Fe protoporphyrin}}{\text{Chlorophyll}} \stackrel{?}{=} 2H_2O$$

In this equation (e) represents electrons of high potential energy contained primarily in the configuration of organic substrates; $-\Delta H$ is the heat liberated in oxidation; and $nh\nu$ is the number of light quanta stored. The forward reaction of oxidation with O_2 to form water takes place with a release of energy; this reaction is catalyzed by a series of heme enzymes which have Fe protoporphyrin as their prosthetic group. The reverse reaction, leading to a storage of energy, is the reaction of photosynthesis. Here the storage of the energy of sunlight is catalyzed by another porphyrin pigment, chlorophyll.

The significance of this scheme of heme and chlorophyll synthesis from the view-point of the evolution of oxidative and photosynthetic mechanisms is discussed elsewhere (16).

SUMMARY

From Chlorella mutant 31 a compound has been isolated with the properties of Mg vinyl pheoporphyrin a_5 . Evidence supporting the identification of this compound is based primarily on spectrophotometric and solubility properties of a number of derivatives prepared from this compound.

Spectrophotometric analysis of the pale green pigment as isolated from ('hlorella indicated that it was a mixture of vinyl pheoporphyrin a_5 and its Mg complex. On the assumption of Mg vinyl pheoporphyrin a_5 and the extinction value at 623 m μ the Mg++ content of an aliquot of the sample was calculated. This agreed with the Mg++ determined directly on the sample by the microcolorimetric method.

Identification of vinyl pheoporphyrin a_{δ} from *Chlorella* was made by comparing the spectrum with the synthetic crystalline compound derived from spinach. The agreement in extinction maxima and wave-lengths of the absorption bands was satisfactory. The vinyl pheoporphyrin a_{δ} compound from *Chlorella* was converted to vinyl chloroporphyrin e_{δ} trimethyl ester and its absorption spectrum agreed in band maxima and wave-lengths with that of the crystalline synthetic compound. The vinyl pheoporphyrin compound from *Chlorella* was reduced with palladium and hydrogen in acetic acid, and the shift in absorption bands supported the view that only one vinyl group was present; the spectrum agreed with that of synthetic vinyl pheoporphyrin reduced at the vinyl group in the same way and with the published data on pheoporphyrin a_{δ} . The compound of *Chlorella* reduced at the vinyl group was converted to an oxime and spectrophotometrically matched the spectrum of the published curve of pheoporphyrin a_{δ} oxime.

Several pigments related to vinyl pheoporphyrin were observed in low concentrations, especially if the *Chlorella* was grown at a high temperature. These pigments appear to be decomposition products of vinyl

pheoporphyrin, possibly vinyl phylloerythrine and vinyl hydroxypheoporphyrin.

Confirmatory evidence supporting the structure of protochlorophyll as a phytyl ester was obtained in solubility studies of the compound isolated from etiolated barley seedlings.

Illumination of *Chlorella* 31 at low temperatures suggests that Mg vinyl pheoporphyrin is destroyed on exposure to light unless it is esterified with a phytol.

A microcolorimetric modification of the Titan yellow method is described for the determination of Mg^{++} in the range of 1 to 4 γ .

We desire to acknowledge the invaluable technical assistance of Miss Lorraine Blake in this study.

BIBLIOGRAPHY

- 1. Granick, S., J. Biol. Chem., 172, 717 (1948).
- 2. Koski, V. M., and Smith, J. H. C., J. Am. Chem. Soc., 70, 3558 (1948).
- 3. Granick, S., and Gilder, H., Advances in Enzymol., 7, 305 (1947).
- 4. Willstatter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin (1913).
- 5. Fischer, H., and Stern, A., Die Chemie des Pyrrols, Leipzig (1940).
- Fischer, H., Mittenzwei, II., and Oestreicher, A., Z. physiol. Chem., 257, p. iv (1939).
- 7. Stern, A., and Wenderlein, H., Z. physik. Chem., Abt. A, 174, 81 (1935).
- 8. Stern, A., and Wenderlein, H., Z. physik. Chem., Abt. A, 176, 81 (1936).
- 9. Noack, K., and Kiessling, W., Z. physiol. Chem., 182, 13 (1929); 193, 97 (1930).
- 10. Fischer, H., and Oestreicher, A., Z. physiol. Chem., 262, 243 (1940).
- Smith, J. H. C., in Franck, J., and Loomis, W. E., Photosynthesis in plants, Ames, 209 (1944).
- 12. Ludwig, E. E., and Johnson, C. R., Ind. and Eng. Chem., Anal. Ed., 14, 895 (1942).
- 13. Lowry, O. H., and Bessey, O. A., J. Biol. Chem., 163, 633 (1946).
- 14. Shemin, D., Cold Spring Harbor Symposia Quant. Biol., 13, 185 (1948).
- 15. Granick, S., J. Biol. Chem., 175, 333 (1948).
- 16. Granick, S., Harvey Lectures, 44 (1948-49) in press.

CHEMICAL DETERMINATION OF HISTAMINE IN HUMAN BLOOD*

By ROSE LUBSCHEZ

(From The New York Hospital and the Department of Pediatrics, Cornell University Medical College, New York City)

(Received for publication, September 24, 1949)

The normal histamine content of human blood as determined by bioassay ranges from 1 to 8 γ per 100 ml., with an average value of about 4 γ (1,2). These low levels in human blood have limited the development of a procedure for chemical analysis, although a chemical method applicable to blood would facilitate the accumulation of data from large groups of subjects.

The two chemical methods available for the analysis of histamine in biological fluids are not directly applicable to human blood. The simpler technique of Rosenthal and Tabor (3), which utilizes the non-specific coupling reaction of imidazoles with 4-nitroaniline, requires a specimen of at least 20 ml. In McIntire's method, the histamine is isolated from plasma under specified conditions (4) and the 2,4-dinitrofluorobenzene derivative is used for the determination (5). This procedure has the required degree of sensitivity but involves two counter-current extraction steps and a scaling down to micro levels for analysis of human blood.

By modifying the purification procedure of McIntire to make it applicable to whole blood filtrates without appreciable loss of histamine, and by scaling down the chemical method of Rosenthal to increase its sensitivity 5-fold, a procedure was developed for the colorimetric analysis of 0.1 to 1.0γ of histamine in a 5 to 10 ml. specimen of whole blood.

EXPERIMENTAL

It has been reported that the histamine of the blood is concentrated in the leucocytes (6). Analysis of white cells or whole blood, therefore, seems preferable to plasma, and, of these, analysis of whole blood is the simpler procedure.

Prior to application of McIntire's method (4) for the isolation of histamine from plasma, it is necessary to precipitate the proteins in whole blood. Several protein precipitants were tested. Trichloroacetic acid gives the clearest filtrates but interferes with subsequent adsorption on the ion exchange medium. Histamine is precipitated by alkaline zinc, barium hy-

* This study was aided by grants from the Commonwealth Fund and the Helen Hay Whitney Foundation.

droxide, and ethyl alcohol. Metaphosphoric acid filtrates contain a small amount of protein but they are satisfactory since the filtered protein is later precipitated during butanol extraction.

In McIntire's method for the purification and concentration of histamine from biological fluids advantage is taken of the fact that histamine is extracted from an aqueous solution by an equal volume of *n*-butanol containing at least 20 per cent by weight of salt at pH 12 or more. Under these conditions, and particularly in plasma, extraction is highly selective for histamine. When whole blood filtrates are treated similarly, other substances, particularly ergothioneine, are also extracted to some extent by butanol and these substances must be treated as interferences in the subsequent analysis.

Histamine is adsorbed from the butanol extract by an ion exchange medium which contains free carboxyl groups. McIntire proposed the use of cotton acid succinate. In our hands the cotton ester was found to vary in efficiency and, for quantitative recoveries, it was necessary to reduce the flow of the butanol solution through the column to an impractical rate. The substitution of Amberlite IRC-50, a resin of the carboxylic acid type which has similar adsorption properties, for the cotton ester was found to facilitate the isolation of histamine. The resin was found to be inefficient in column operation but quantitative adsorption was obtained in batch operation by shaking the resin beads with the butanol solution for a specified time. Neither cotton acid succinate nor Amberlite adsorbs histamine when more than trace amounts of inorganic salts are present. Contamination of the butanol extract by the aqueous phase must, therefore, be avoided. Prevention of losses during the elution step due to the solubility of histamine in water and the presence of traces of inorganic salts on the resin beads is achieved by washing the resin free of butanol in 95 per cent ethyl alcohol alone.

It was found that the quantitative elution of histamine from the Amberlite required the eluting acid to be in contact with the resin for 15 minutes. Subsequent rinsing of the acid with sodium nitrite, necessary for the chemical analysis, provided conditions for the complete elution of histamine. The separate elution step is necessary, since the resin itself otherwise releases a large amount of material which produces color during the subsequent chemical analysis.

Rosenthal's method (3) is sufficiently sensitive for detection of $0.5~\gamma$ of histamine in pure solution as the red azo compound which results from the coupling of 4-imidazole ethanol with the diazotate of 4-nitroaniline. For our purposes it was necessary to reduce the volumes and change the proportions of the reagents in order to obtain reproducible readings in the range from 0.1 to $0.5~\gamma$.

Quantitative determination of these small amounts of histamine in whole blood is complicated by several sources of interference. The copper present in whole blood, presumably because it is bound to protein, does not interfere with the determination, although copper ion inhibits the coupling reaction. The fact that histamine added to intact whole blood is not recovered has been attributed to the buffer action of intact blood proteins on a wide variety of active substances, of which histamine may be one. It was found that the prior treatment of whole blood with a small amount of concentrated sulfuric acid permitted quantitative recoveries of added histamine. This acid was, therefore, added routinely to all blood specimens to prevent protein binding and to inhibit bacterial growth.

Other interfering substances which are extracted from whole blood filtrates by butanol are not removed by an initial extraction with ether and are also adsorbed by the ion exchange medium and eluted under the same conditions as histamine. These include large amounts of a number of blood extractives. Analysis of whole blood from the pig failed to reveal histamine, although at least one other compound, presumably ergothioneine, was extracted and gave appreciable color on analysis. Such interference is minimized by prolonged heating of the cluate with a higher concentration of nitrous acid than was originally suggested by Rosenthal.

Although butanol extraction removes some substances which interfere with the coupling reaction, and heating with nitrous acid minimizes the effect of other interfering substances present in the butanol extract, extraneous color still develops during the coupling reaction. Rosenthal found on analysis that of the substances in the blood filtrates which couple with 4-nitroaniline only the azo compound of histamine retains its red color at pH 7.7, while the other azo compounds range in color from yellow to orange at this pH. Blood which had been stored in the blood bank for 1 month and found to be free of histamine yielded a yellow azo compound at pH 7.7 on analysis. This yellow color showed no change over a pH range from 7.7 to 3.0. In contrast, solutions containing only the azo compound of pure histamine showed a color range from red to orange to yellow over a pH range of 7.7 to 4.0.

These color changes were utilized to correct for the extraneous color developed during the blood analysis. The tubes were read at pH 7.7, i.e. after equilibration with veronal buffer, at which only histamine produced a red color, and at pH 3.0, obtained by the addition of a measured drop of glacial acetic acid, at which all the reaction products were yellow. The difference between the optical densities of the two readings was assumed to be proportional to the amount of histamine present. Differences in optical density plotted against concentration of histamine standards gave good agreement with Beer's law. Both readings were made at a wave-

length of 525 m μ , at which a maximum difference between red and yellow absorption occurs.

Reagents—All reagents must be made up in copper-free water.

- 1. Metaphosphoric acid, approximately 6 per cent. Prepare fresh each week and store in the refrigerator.
- 2. Sodium nitrite, 4 and 1.6 per cent. Prepare fresh every week and store in the refrigerator.
- 3. 4-Nitroaniline, 2 per cent. Dissolve 2.0 gm. of 4-nitroaniline in 100 ml. of 4 per cent (by volume) HCl. Store in the refrigerator.
- 4. Diazonium working solution. Add 0.5 ml. of 4 per cent sodium nitrite to 5 ml. of cold nitroaniline solution in an ice bath immediately before use.
 - 5. Sodium carbonate. 21.4 gm. of anhydrous salt per 100 ml. of solution.
- 6. Veronal buffer. 9.2 gm. of diethylbarbituric acid and 2.0 gm. of NaOH per liter, adjusted to pH 7.7 with molar acetic acid.
- 7. Dry salt mixture. Mix anhydrous sodium sulfate and tertiary sodium phosphate monohydrate (4) in a ratio of 6.25 to 1 gm.
- 8. Standard solutions. Solutions of histamine dihydrochloride in 3.8 per cent (by volume) HCl equivalent to concentrations of 0.2, 0.5, and 0.8 γ of free base per ml. These should be prepared fresh for each determination from a stock solution containing 1 mg. of free base per ml. in 3.8 per cent HCl. The stock solution may be stored for 1 month in the refrigerator.
 - 9. HCl. 3.8 per cent by volume of concentrated (37.5 per cent) HCl.
 - 10. Amberlite IRC-50.2
 - 11. NaOH, 20 per cent.
- 12. n-Butanol, reagent grade. Redistil if a blank gives color with the diazonium reagent.
 - 13. Methyl isobutyl ketone, commercial purified grade.

Procedure

It is necessary to use calibrated constriction pipettes for all measurements of volumes of 1 ml. or less. These may be made or may be purchased from the Microchemical Specialties Company, Berkeley, California.

¹ The commercial c.p. acid in pellet or stick form contains a minimum of 25 per cent sodium phosphate and is not as effective a protein precipitant as acid prepared by dehydrating c.p. orthophosphoric acid. For the preparation of metaphosphoric acid 300 ml. of 85 per cent orthophosphoric acid are heated in a 1 liter graphite crucible until the water is drawn off and fumes of phosphorus pentoxide appear. The crucible is heated for 2 additional hours. The contents are poured into a metal container with a tight fitting cover and allowed to cool. This preparation does not solidify.

² Obtained through the courtesy of the Resinous Products Division, Rohm and Haas Company, Philadelphia.

Concentration of Histamine from Whole Blood—A 5 to 10 ml. sample of oxalated blood, collected under sterile precautions, is measured and delivered into a sterile tube. 1 drop (0.01 ml.) of concentrated sulfuric acid is added for each 2 ml. of blood and the contents are mixed. In this condition the blood may be analyzed immediately or stored at -15° . The blood is transferred to a 50 ml. round bottomed centrifuge tube and the storage tube is rinsed with an equal volume of water and 2 volumes of metaphosphoric acid. The rinsings are added to the blood, giving approximately a 1:4 dilution. After thorough mixing the tube is centrifuged at 2500 r.p.m. (radius 30 cm.) for 15 minutes, and the clear supernatant solution is poured into a 100 ml. round bottomed centrifuge tube equipped with a ground glass stopper. The solution is made alkaline to nitrazine paper with 2 to 5 ml. of 20 per cent NaOH, and 1 gm. of dry salt mixture is added for each 3.75 ml. of the supernatant solution. A volume of n-butanol equal to the total alkaline solution is added. The tube is stoppered and shaken mechanically for 30 minutes. After centrifugation at 1500 r.p.m. to separate the layers, most of the butanol layer is transferred to another glass-stoppered tube containing 400 mg. of dry Amberlite IRC-50. The aqueous layer is extracted a second time with butanol and the extracts are combined. Removal of the last traces of the second butanol layer is facilitated by the addition of several 5 ml. portions of fresh butanol. Contamination of the butanol extract by the aqueous phase should be avoided. The combined butanol extracts are shaken mechanically for 1 hour with the Amberlite. All of the Amberlite is then transferred to a 140 \times 21 mm. tube with a narrowed section 25×9 mm. above a stop-cock and burette tip. The stop-cock opening is covered by a small plug of glass wool. The transfer is best accomplished by pouring the butanol through the tube, the separated butanol being used to rinse the Amberlite into the The tube and Amberlite are rinsed once with about 40 ml. of 95 per cent ethyl alcohol which is drained by applying gentle suction to the tip of the tube. The stop-cock is closed and 1 ml. of 3.8 per cent HCl is delivered to the Amberlite and allowed to stand in contact with it for 15 minutes. The acid is drained dropwise into a 12×75 mm. Pyrex tube. The Amberlite is then rinsed with 1.0 ml. of 1.6 per cent sodium nitrite, which is also delivered into the Pyrex tube. The last drops of rinse solution are blown into the Pyrex tube.

Analysis of Histamine—The tube containing the Amberlite eluate is heated in an oven at 100° for 30 minutes. At this stage the tubes may be stoppered and stored overnight in the refrigerator, or chilled in an ice bath and analyzed immediately. To the cold Amberlite eluate in an ice bath, 0.2 ml. of the diazonium reagent is added with mixing, followed by 0.4 ml. of sodium carbonate. The contents are mixed and allowed to stand for

3 minutes. 0.25 ml. of methyl isobutyl ketone is pipetted into the tube and the contents are shaken vigorously thirty times, allowed to separate in the ice bath, and the tube is finally centrifuged. By means of a capillary pipette, the largest possible portion of uncontaminated ketone layer is transferred to another 12×75 mm. tube containing 1 ml. of veronal buffer measured by burette. The ketone is shaken with the buffer twenty times and allowed to stand in contact with it for 30 minutes in the refrigerator with occasional shaking. The ketone layer is then transferred to a 6 \times

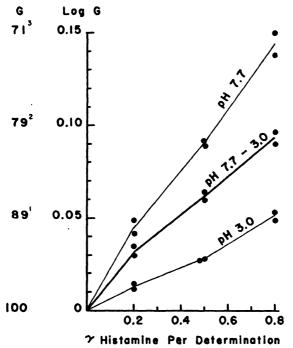


Fig. 1. Analysis of standard histamine solutions. G = galvanometer reading.

75 mm. colorimeter tube³ and centrifuged if not perfectly clear. The tube is read in the Coleman junior spectrophotometer at a wave-length of 525 m μ against a blank prepared similarly with 1.0 ml. of 3.8 per cent HCl. For the standard curve, 1 ml. volumes of standard histamine solutions containing 0.2, 0.5, and 0.8 γ per ml. are carried through the analysis in duplicate at the same time. Following these readings, 1 measured drop

³ These tubes are made from Kimble hydrometer glass tubing stock, 6 mm. outside diameter, wall thickness less than 1 mm. to fit the Coleman adapter No. 6-110. The slit in this adapter is partially filled with Plicene cement so that a volume of 0.2 ml. of ketone may be read.

(3 to 5 c.mm.) of glacial acetic acid is added to each tube and the contents are mixed. The tubes are again read at 525 m μ . The concentration of histamine is proportional to the difference between the optical densities of the two readings.

Table I
Recovery of Added Histamine from 10 Ml. of Histamine-Free Blood

Added histamine	Found on analysis	Per cent recovery
γ	γ	
0.2	0.20	100
0.2	0.19	95
0.5	0.46	92
0.5	0.51	102
0.8	0.74	93
0.8	0.81	101
1.0	0.95	95
1.0	1.01	101

Table II

Chemical Determination of Histamine Levels in Human Blood

Subject	Age	Volume of specimen	Total histamine content	Hista- mine level	Comment
		ml.	γ	γ per 100 ml.	
HN	Adult	10	0.16	1.6	Normal
		10	0.14	1.4	u
NE	"	9	0.40	4.4	u
		9	0.42	4.6	"
RL	"	7	0.30	4.3	"
		7	0.26	3.7	"
PG	"	10	0.06	0.6	"
		10	0.08	0.8	"
760	66	10	0.27	2.7	Asthma
	yrs.				
750	11/2	7	0.49	7.0	Infantile eczema
751	$2\frac{1}{2}$	9	0.82	9.1	" "
752	11	9.5	0.07	0.7	Normal
823	91	12	0.42	3.5	. "

Results

In Fig. 1, the curve obtained on analysis of standard solutions of histamine is presented together with the logarithms of the colorimetric readings at pH 7.7 and 3.0.

In Table I the percentage recovery of standard solutions of histamine

from 10 ml. of acidified blood is given. The blood used had been stored for at least 1 month and had been found to be free of histamine on analysis. Recoveries ranged from 92 to 102 per cent.

In Table II, blood histamine levels in micrograms per 100 ml. of blood are given for nine subjects. The four duplicate determinations show a reproducibility within 10 per cent. Of the nine determinations, the two values higher than 5γ per 100 ml. were obtained in children with infantile eczema whose white cell counts were at the upper limit of normal. The significance of the wide range in levels below 5γ per 100 ml. is unknown.

DISCUSSION

In a limited series of subjects, a wide range in blood histamine was found (0.1 to 9.1 γ per 100 ml.). The values obtained for normal subjects fell within the lower limits of normal reported by bioassay (1); two patients with active allergy had blood levels at the upper limit of the normal. These findings are consistent both with the reported results of bioassay and with the observation that patients with allergic manifestations have high blood histamine levels (7). A study of the blood histamine levels in a large series of children with various conditions is now in progress.

Probably whole blood histamine alone is measured by the proposed method. It is less sensitive but more easily reproducible than methods based on bioassay. With careful technique, its reproducibility is within 10 per cent. The lack of analytical precision is compensated for in part by the fact that the chemical analysis permits storage of specimens over a long period of time and the procedure is less time-consuming.

SUMMARY

The histamine purification method of McIntire and the chemical method of Rosenthal and Tabor have been modified to permit the analysis of 0.1 to 1.0 γ of histamine in human blood samples of 5 to 10 ml., with an error of 10 per cent. The levels obtained by this method in a small group of subjects are reported.

BIBLIOGRAPHY

- 1. Rose, B., and Browne, J. S. L., Proc. Soc. Exp. Biol. and Med., 44, 182 (1940).
- 2. Haworth, E., and MacDonald, A. D., J. Hyg., 37, 234 (1937).
- 3. Rosenthal, S. M., and Tabor, H., J. Pharmacol. and Exp. Therap., 92, 425 (1948).
- 4. McIntire, F. C., Roth, L. W., and Shaw, J. L., J. Biol. Chem., 170, 537 (1947).
- 5. McIntire, F. C., Sproull, M., and White, F. B., Federation Proc., 8, 102 (1949).
- 6. Code, C. F., J. Physiol., 90, 485 (1937).
- 7. Rose, B., J. Clin. Invest., 20, 419 (1941).

THE EFFECT OF A SERIES OF FLAVONOIDS ON HYALURONI-DASE AND SOME OTHER RELATED ENZYMES*

By GERTRUDE RODNEY, ANN L. SWANSON, L. M. WHEELER, GRANT N. SMITH, AND CECILIA S. WORREL

(From the Research Laboratories of Parke, Davis and Company, Detroit)

(Received for publication, August 29, 1949)

Among the theories which have been advanced for the possible mechanism of action of vitamin P substances in regulating capillary resistance, it has been proposed (1) that they may act as inhibitors of hyaluronidase. As evidence that this enzyme may play a part in capillary permeability, the capillary wall and supporting tissue have been reported (2, 3) to contain metachromatic staining material, either hyaluronic acid or chondroitin, both of which are subject to attack by hyaluronidase. Duran-Reynals (4) showed that the enzyme increased the permeability of the vascular system, and Chambers and Zweifach (5) found petechial hemorrhages following injection of hyaluronidase in mesentery preparations.

The effect of a limited number of flavonoids on the action of hyaluronidase in vitro was studied by Beiler and Martin (1) and by Clark and Geissman (6). Levitan (7) and Elster (8) have reported on the action of rutin on hyaluronidase in vivo. The present report deals with the effect of thirteen flavonoids on hyaluronidase, with two methods in vitro and one test in vivo, in a study of the relation of structure to inhibitory activity. In addition, the action of these compounds on some other enzymes involved in theories of vitamin P activity is reported in order to determine the specificity of the inhibitory activity.

Streptococcal hyaluronidase differs from the bovine enzyme in its sensitivity to pH changes, optimum pH of 7.0, and greater release of reducing substances from the substrate (9). It was chosen for study as being closely related to the bovine enzyme. β -Glucuronidase, which releases glucuronic acid from conjugates but has no action on hyaluronic acid, was also studied with the flavonoids. Lysozyme, an enzyme which liberates ketohexose and hexosamine from polysaccharides of certain bacteria but not from hyaluronic acid, was used as a third related enzyme. In addition, the effects of the compounds on the oxidation of epinephrine by mushroom tyrosinase were investigated, since inhibition of epinephrine oxidation has been suggested as the basis for vitamin P action (10).

^{*} Presented at the meeting of the Federation of American Societies for Experimental Biology, Detroit, April, 1949.

Materials and Methods

Inhibitors—The thirteen flavonoids which were studied differed in the state of oxidation, the number of hydroxyl groups, the sugar components, and the methoxyl groups. Suspensions of 1 mg. of flavonoid per ml. of M/15 phosphate buffer, pH 7.5, were shaken, left at room temperature for 24 hours, centrifuged if necessary, and the concentration determined by the spectrophotometric method. The solutions were diluted with buffer to a concentration of 1×10^{-4} mole per ml., except in the case of rhamnetin, which was soluble only to the extent of 3×10^{-5} mole per ml. Dilutions of quercetin and xanthorhamnin were made to compare with this, since their structural relationship was important.

These solutions were tested by both *in vitro* methods. In addition, in the viscosity test, in which a much smaller volume of inhibitor and enzyme could be used, they were dissolved in 1 per cent tris(hydroxymethyl)aminomethane (TAM). Control experiments showed that this solvent with pH 9.0 had little effect on the test. For the work *in vivo* the flavonoids were injected at pH 8.0 or in 1 per cent TAM with appropriate controls.

Bovine Hyaluronidase—The enzyme was prepared by the Madinaveitia method (11) and contained 200 viscosity reducing units per mg. (400 turbidity reducing units per mg.). The substrate, hyaluronic acid, was prepared from umbilical cords by the Dorfman method (12). It contained 13.5 per cent glucosamine, 45.5 per cent reducing substances in terms of glucose, and 3.0 per cent nitrogen.

For testing the effect of the flavonoids on viscosity reduction by the enzyme, the Haas method (13) was used; 100 viscosity reducing units of enzyme were incubated 1 hour with 10 ml. of flavonoid solution, and enzyme diluted with the same amount of solvent was used as a control. After determining the initial flow time of 4 ml. of substrate (1.5 mg. per ml. of Haas buffer) in an Ostwald viscosimeter at 25°, 0.1 ml. of the enzyme solution was added to the substrate, and at 2 minute intervals the flow time was redetermined. The inhibition was calculated from the formula, 2 per cent inhibition = $100 \times 1 - y - x/y - z$, where y = the initial flow time of enzyme, substrate, and inhibitor, x = the flow time after 10 minutes for the enzyme, the substrate and inhibitor, and z = the

¹ Dihydroquercetin, eriodictyol, homoeriodictyol, rhamnetin, and xanthorhamnin were supplied by Dr. W. G. Bywater of S. B. Penick and Company. Naringin, hesperidin, and hesperidin methyl chalcone were supplied by the California Fruit Growers Exchange. The remaining flavonoids studied were prepared by one of us (L. M. W.).

² It was not possible to determine inhibition by the formula (100 \times R - R_0)/R, since at the concentrations of inhibitors used, the point of the half viscosity could not be reached with many of the compounds.

flow time after 10 minutes for the enzyme and the substrate. Additional controls were run with appropriate dilutions of the flavonoid, substrate, and buffer.

A modification of the Dorfman method (12) was used for testing the effect of the inhibitors on the turbidity-reducing action of the enzyme. 10 units of the hyaluronidase in 0.5 ml. of 0.9 per cent saline were mixed with 0.5 ml. of the flavonoid solution and let stand 1 hour. 1 ml. of substrate containing 0.2 mg. of hyaluronic acid in 0.1 m acetate buffer at pH 6.0 was added, and the mixture was incubated 30 minutes at 37°. After heating the tubes at 60° for 10 minutes, 8 ml. of 0.1 per cent Fraction V bovine albumin in acetate buffer at pH 3.75 were added to develop the turbidity of the residual hyaluronic acid, and the tubes were read in a Fisher electrophotometer at 525 m μ . Controls were run with inhibitors and substrate, and the per cent inhibition was calculated by comparison with the activity of the enzyme and the substrate alone.

The *in vivo* method was that of Humphrey (14), with modifications according to Hechter and Sully (15). Ten 300 gm. white guinea pigs were injected 4 hours before the test with flavonoids dissolved at pH 8.0 in an amount, or a fraction thereof, equimolar with 100 mg. of rutin. The animals were shaved and injected on each side with 0.2 ml. of a control solution of hemoglobin and saline, prepared by hemolyzing packed rabbit erythrocytes with 5 volumes of water, centrifuging, and diluting 1:1 with 0.9 per cent saline. They were also injected on each side with 0.2 ml. of hemoglobin saline containing 0.01 unit of hyaluronidase. Normal animals were similarly treated. After 20 minutes they were killed, and the areas of spread on the inner surface of the skin were calculated by multiplying the product of two diameters measured at right angles by $\pi \div 4$. The average values for the spread with saline were subtracted from those with enzyme, and the per cent inhibition was determined by comparison with the values for normal animals.

Streptococcal Hyaluronidase—The enzyme was prepared from filtrates of streptococcus type B grown in Difco brain-heart infusion for 4 days at 37°, and fractionated at pH 7.2 with (NH₄)₂SO₄. The fraction between 35 and 70 per cent of saturation was dialyzed and lyophilized to yield material with 15 viscosity-reducing units per mg. The hyaluronic acid used as the substrate was prepared by the method given previously; the testing procedures and solutions of the flavonoids were the same as in the studies with the bovine enzyme.

β-Glucuronidase—The procedure of Graham (16) was followed for the preparation of the enzyme, which contained 1700 units per mg. of N, as determined by the method of Fishman, Springer, and Brunetti (17) with phenolphthalein glucuronide as the substrate.

Solutions of the flavonoids in m/15 phosphate buffer were diluted to

 1×10^{-4} mole per ml. with water, and to 1 ml. of these solutions 0.1 ml. of 1:10 glucuronidase and 0.8 ml. of acetate buffer, pH 4.5, were added. They were incubated at room temperature for 30 minutes and 0.1 ml. of 0.01 m phenolphthalein glucuronide solution was added. After incubating 1 hour at 37°, the reaction was stopped by addition of 2.5 ml. of 0.4 m glycine buffer at pH 10.5. To the control tubes 1 ml. of the inhibitor solutions was then added, and after 1 minute they were read in the Fisher colorimeter at 525 m μ and compared with the standard phenolphthalein curve in the usual way.

Lysozyme—Crystalline lysozyme was used for the study; the substrate was a suspension of Micrococcus lysodeikticus cells (18). To 4 ml. of this suspension 1 ml. of the flavonoid solutions (1 \times 10⁻⁴ mole per ml.) was added and the initial turbidity was determined in the Fisher electrophotometer at 525 m μ . A 0.2 ml. sample of lysozyme, 0.2 mg. per ml. of 0.85 per cent saline, was then introduced, and the contents of the tube were mixed by inverting ten times. The decrease in optical density in 2 minutes was then estimated and compared with blank determinations containing no flavonoids.

Epinephrine Oxidation by Tyrosinase—The enzyme was prepared from mushrooms by the method of Parkinson and Nelson (19) and contained 4 p-cresolase units per mg. It was dissolved in water, 2 mg. per ml., and to 1 ml. of enzyme in Warburg vessels 1 ml. of the flavonoids (1 × 10⁻⁴ mole per ml.) was added. After equilibration 0.5 ml. of 0.02 m epinephrine was added. Controls were run with flavonoid and enzyme and flavonoid and epinephrine, as well as with epinephrine and enzyme and epinephrine alone. Flasks were run in duplicate and the experiments were carried out in air at 37°. The per cent inhibition was determined after subtracting the small values due to autoxidation of the epinephrine and epinephrine-flavonoid mixtures and to the oxidation of the flavonoid by the enzyme, so that the values obtained represented the true inhibition of the enzyme.

Results

The effects of the flavonoids on hyaluronidase are shown for the four tests in Table I. The results of the *in vitro* tests indicate that the aglucones are more inhibitory than the glucosides, since quercetin was more active than rutin or quercetrin, rhamnetin more active than xanthorhamnin, and naringenin more active than naringin. A methyl group blocking hydroxyls in the 3' position reduced the activity; homoeriodictyol was much less inhibitory than eriodictyol. However, a methoxyl group in the 7 position had little effect, since rhamnetin and quercetin were equally active. Hydroxyls in both the 3' and 4' positions produced greater activity than a single hydroxyl, as evidenced by the greater activity of

eriodictyol compared with naringenin. The presence of a ketone group did not appear to be essential.

With increased alkalinity (as in TAM), activity was shown by some compounds which were inactive at lower pH. It is known that an increased oxidation of catechol takes place in alkali, and it was observed that aging the solutions in air produced increased activity and a darkening of color in many instances, so that the state of oxidation has considerable influence.

Table I

Effect of Flavonoids on Bovine Hyaluronidase

	Per ce	nt inhibition	in vilro	Inhibition
Compound	Turbidity,	Visc	Compared	
	pH 7 5	pH 75	1 per cent TAM	with rutin, mole basis
Rutin	0	0	54	1.0
Quercetrin	0	5	66	0.8
Quercetin	33, 17*	44, 20 *	65	1.0
Dihydroquercetin	44	37	64	2.6
D-Catechin	. 88	70	73	6.3
Eriodictyol	100	85	85	2.0
Homoeriodictyol	0	0	23	0.0
Xanthorhamnin	0, 0*	0, 0*	23, 10*	0.5
Rhamnetin	11*	12*	22*	0.3
Naringin	0	0	14	0.8
Naringenin	6	0	35	
Hesperidin	0	0	0	0.0
" methyl chalcone	0	0	0	

^{*} 3×10^{-5} mole per ml

In the *in vivo* test the aglucones had no greater activity than the glucosides, as might be expected, since the sugar components could easily be removed. Here also the compounds most active *in vitro* and most susceptible to oxidation showed the greatest inhibition. Several compounds which were inactive *in vitro* showed activity *in vivo*, which would be compatible with the theory that activation by oxidation in the tissues could take place.

In an attempt to clarify these *in vivo* results, the effect of some tissues and enzymes on the activation of the inhibitory action of these compounds was studied. A suspension of 100 mg. of rutin was incubated 24 hours with hog pancreas homogenate (1 gm. per 10 ml. of m/15 phosphate buffer, pH 7.5). After removal of protein by heating 20 minutes, followed by cen-

trifuging, the supernatant was tested for inhibitory activity. Appropriate controls with heated pancreas homogenate and heated rutin were also run. Some slight evidence of activation was found, but the results were very variable, and similar experiments with pancreatin, taka-diastase, emulsin, and muscle homogenate gave negative results. However, which liver, spleen, and blood were tested under the same conditions as those described for pancreas homogenate, slight but constant activation was produced (Table II).

Since it is known that catechol and related compounds are rapidly oxidized by peroxidase in the presence of traces of H_2O_2 in tissues, the effect of a purified preparation of this enzyme was studied. The enzyme was isolated from horse-radish and in solution contained 0.2 mg. of N per ml.; 1 ml. was equivalent to 1.75 mg. of purpurogallin. 2 ml. of fresh

Table II

Effect of Tissues or Enzymes on Rutin Inhibition of Hyaluronidase

Tissue or enzyme	Per cent inhibition
Pancreas	19, 0, 7, 0
Pancreatin	0, 0
Taka-diastase	0, 0
Emulsin .	0, 0
Muscle homogenate	0, 0
Liver "	10, 17
Spleen "	15, 25
Whole blood	20, 25
Peroxidase	50

solutions of the inhibitors were treated with 0.01 ml. of 1:100 $\rm H_2O_2$ and 0.1 ml. of peroxidase for 30 minutes. The reaction was stopped by the addition of 0.5 ml. of 2 N $\rm H_2SO_4$. After neutralizing and warming to remove any residual peroxide, they were tested for inhibitory action by both of the *in vitro* methods, with appropriate controls. The results are shown in Table III. Definite activation of naringenin, rutin, and dihydroquercetin was produced, but none for hesperidin.

The results of the study of the action of flavonoids on the other systems are shown in Table IV. It is apparent that those compounds most inhibitory to bovine hyaluronidase were also most active against the four enzymes investigated here, while those with least activity against the bovine enzyme had negligible inhibitive effect in these systems. Some difference in degree of action can be observed: β -glucuronidase was inhibited to some extent by all the compounds tested, though the greatest action was shown by quercetin, D-catechin, dihydroquercetin, and eriodictyol, as with the

other systems. The streptococcal hyaluronidase also was most strongly affected by these compounds.

Table III

Effect of Peroxidase on Flavonoid Inhibition of Hyaluronidase

Compound	Treatment	Per cent inhibition	
	Treatment	Turbidity	Viscosity
Rutin		0	0
	Peroxidase	54	16
Dihydroquereetin		30	42
•	Peroxidase	100	67
Naringenin		0	0
5	Peroxidase	50	27
Hesperidin		0	0
•	Peroxidase	0	0

TABLE IV

Effect of Flavonoids on Enzymes

Per cent inhibition

rer cent indiction					
Compound	Streptococcal hyalu- ronidase		β-Glucu- ronidase	Lysozyme	Tyrosin-
	Turbidity	Viscosity	Tomase		
Rutin	0	0	28	0	17
Quercetrin	0	0	15	0	
Quercetin	90	39	38 9*	15	11
Dihydroquercetin	100	36	51	36	72
D-Catechin	100	73	39	64	35
Eriodictyol .	100	59	26	33	41
Homoeriodictyol	0	6	6	0	0
Xanthorhamnin	0 0*	0.0*	14.3*	6.4*	
Rhamnetin	0*	0*	15*	15*	
Naringin	0	0	7	0	7
Naringenin	7	0	28	10	31
Hesperidin	0	0	13	0	0
" methyl chalcone	0	0	25	0	

^{*} 3×10^{-5} mole per ml.

DISCUSSION

Recently James and coworkers (20) have shown that catechol, with free orthohydroxyl groups, is oxidized to the orthoquinone form with the introduction of a third hydroxyl group, active in forming color complexes and reacting with amino groups of protein. Resorcinol, with metahy-

droxyl groups, does not undergo this reaction. In the case of naringenin the introduction of a hydroxyl group in the 3' position, already present in rutin and dihydroquercetin, would lead to quinone formation and further oxidation, but with hesperidin the probable reactive position 5' would place hydroxyls in the meta position. This presents a possible explanation of the oxidative activation of the inhibitory action on hyaluronidase for those flavonoids with structures capable of forming orthoquinones. A similar reaction has been discussed by Bartlett (21) in connection with the inhibition of succinoxidase by the action of some orthoquinones on sulf-hydryl groups of the enzyme. Hyaluronidase is inhibited by p-chloromercuribenzoate, a sulfhydryl-reactive compound, and contains free amino groups, so that it should be susceptible to the reactions discussed.

The *in vivo* results may be explained on the basis of oxidation by tissues and blood. In addition to other oxidative systems blood contains peroxidase; spleen and liver are blood-rich organs and these tissues were found to have an activating effect on rutin. The erratic results with pancreas may be explained by variations in the amount of blood present in the preparation.

From the results obtained in the studies of the action of the flavonoids on five enzymes, it appears that all of the enzymes were inhibited by those compounds capable of orthoquinone formation and that inhibition by the flavonoids is not specific, but probably due to reaction with amino or thiol groups in the protein molecule.

SUMMARY

Thirteen flavonoids were tested for their inhibitory action on bovine hyaluronidase by *in vitro* and *in vivo* methods. For maximum activity *in vitro* the aglucones were more effective than the glucosides, and free hydroxyl groups in both the 3' and 4' positions were necessary. Activation by some tissues, by increased pH, and by oxidation such as by peroxidase, may explain the *in vivo* activity of many of the flavonoids against hyaluronidase.

The inhibitory action of these flavonoids on streptococcal hyaluronidase, β -glucuronidase, lysozyme, and tyrosinase oxidation of epinephrine in general followed the structural pattern observed with bovine hyaluronidase and indicated that the inhibition is not specific.

BIBLIOGRAPHY

- 1. Beiler, J. M., and Martin, G. J., J. Biol. Chem., 171, 507 (1947).
- 2. Cella, L. J., and Means, J. A., Marquette Med. Rev., 13, 67 (1948).
- 3. Bunting, H., and Wislocki, G. B., Am. J. Anat., 81, 1 (1947).
- 4. Duran-Reynals, F., Yale J. Biol. and Med., 11, 601 (1939).

- 5. Chambers, R., and Zweifach, B. W., Physiol. Rev., 27, 436 (1947).
- 6. Clark, W. G., and Geissman, T. A., J. Pharmacol. and Exp. Therap., 95, 363 (1949).
- 7. Levitan, B. A., Proc. Soc. Exp. Biol. and Med., 68, 566 (1948).
- 8. Elster, S. K., Proc. Soc. Exp. Biol. and Med., 71, 15 (1949).
- 9. Rogers, H. J., Biochem. J., 42, 633 (1948).
- 10. Lavollay, J., Compt. rend. Acad., 219, 318 (1944).
- 11. Madinaveitia, J., Biochem. J., 35, 447 (1941).
- 12. Dorfman, A., and Ott, M. L., J. Biol. Chem., 172, 367 (1948).
- 13. Haas, E., J. Biol Chem., 163, 63 (1946).
- 14. Humphrey, J. H., Biochem. J., 37, 177 (1943).
- 15. Hechter, C., and Sully, E. L., J. Exp. Med., 86, 19 (1947).
- 16. Graham, A. F., Biochem. J., 40, 603 (1946).
- 17. Fishman, W. H., Springer, B., and Brunetti, R., J. Biol. Chem., 173, 449 (1948).
- 18. Smith, G. N., and Stocker, C., Arch. Biochem., 21, 383 (1949).
- 19. Parkinson, G. G., Jr., and Nelson, J. M., J. Am. Chem. Soc., 62, 1693 (1940).
- James, W. O., Roberts, E. A. H., Beevers, H., and deKock, P. C., Biochem. J., 43, 626 (1948).
- 21. Bartlett, G. R., J. Pharmacol. and Exp. Therap., 93, 329 (1948).

SYNTHESIS OF HEME IN VITRO BY THE IMMATURE NON-NUCLEATED MAMMALIAN ERYTHROCYTE*

By IRVING M. LONDON, DAVID SHEMIN, AND D. RITTENBERG

(From the Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

(Received for publication, October 24, 1949)

When the whole blood of subjects with sickle-cell anemia is incubated aerobically with N¹⁵-labeled glycine, labeled heme is formed (2). finding demonstrates the synthesis of heme from glycine in vitro. Significant synthesis of heme is not observed when normal human blood is incubated under similar conditions. This difference could be due to the presence of numerous immature cells in the blood in sickle-cell anemia. However, in the initial experiments previously reported, in which the capacity for in vitro synthesis of heme from blood samples from other subjects with hematologic disorders, characterized by elevated reticulocyte counts, was studied, significant heme synthesis was not observed. It was clear, therefore, that, although the reticulocytes in the blood of sickle-cell anemia subjects might be involved in the heme synthesis observed, the mere presence of numerous reticulocytes does not insure the ability of blood to synthesize heme in vitro. These studies have been extended further to a number of disorders in subjects with elevated reticulocyte counts in the peripheral blood. The results of these studies, to be reported in detail later, confirm the finding that some blood samples with elevated reticulocyte counts do not perform significant heme synthesis in vitro; they reveal, however, that the in vitro synthesis of heme in human blood is not restricted to the blood of sickle-cell anemia.

In order to investigate some of the problems posed by these observations, further information on the rôle of the reticulocyte in the synthesis of heme was sought. It was desirable to determine the capacity for synthesis of heme of reticulocytes in the blood of experimental animals which had no previous hematologic disorder. This report is concerned with the capacity for heme synthesis of blood samples drawn from rabbits in which reticulocytosis was produced in response to bleeding or phenylhydrazine hemolysis.

^{*} This work was communicated in part to the American Society for Clinical Investigation at Atlantic City, May, 1949 (1). It was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

EXPERIMENTAL

Normal rabbits were bled by cardiac puncture. 20 to 30 ml. of blood were removed three to five times a week and equivalent volumes of normal saline were injected intraperitoneally. Reticulocyte counts were performed on smears of blood drawn from ear veins and stained with brilliant cresyl blue; 1000 erythrocytes were counted for each determination of the percentage of reticulocytes.

In a typical experiment, 20 to 30 ml. of blood were added to a flask containing heparin, 50 mg. of glycine labeled with 32 atom per cent excess N¹⁵, and 1 to 2 mg. each of crystalline penicillin and streptomycin to inhibit bacterial contamination. The flasks were incubated for 24 hours at 37°; the blood was aerated with a 95 per cent O₂-5 per cent CO₂ mixture. At the end of the period of incubation, the blood was centrifuged and the serum removed. Hemin was prepared from the red cells by the usual procedure (3), recrystallized (4), and its N¹⁵ concentration determined.

Similar experiments were performed with the blood of rabbits in which reticulocytosis was produced by acetylphenylhydrazine. In most instances 60 mg. of acetylphenylhydrazine were injected subcutaneously in one dose several days prior to withdrawal of the blood sample tested. In Experiments 80-2, 80-3, 80-5, 80-9 in Table II, 25 mg. of acetylphenylhydrazine were injected daily for 6 days and blood samples were drawn on the 7th day.

The effect of anaerobiosis was studied in Experiment 74. For 45 minutes prior to the addition of N¹⁵-labeled glycine and for 15 minutes thereafter a mixture of 95 per cent N₂-5 per cent CO₂ was passed through the flask containing the blood sample. The flask was then sealed. The procedure was otherwise identical with that described for the aerobic experiments.

Synthesis of heme by erythrocytes washed free of plasma was investigated in Experiment 80-3 (Table II). After an initial separation of the erythrocytes from the plasma by centrifugation, the cells were washed twice with physiologic saline and were then suspended in 15 ml. of physiologic saline. The incubation with labeled glycine was then carried out as described above.

The capacity for synthesis of heme of erythrocytes hemolyzed by saponin was tested in Experiments 72 and 80-5. In Experiment 72, the erythrocytes were separated from the plasma by centrifugation, washed once with physiologic saline, and then mixed with a solution of 200 mg. of saponin dissolved in 15 ml. of physiologic saline. Hemolysis was complete within 15 minutes. In Experiment 80-5, the erythrocytes were washed twice with physiologic saline and were then mixed with a solution of 37.5 mg. of saponin dissolved in 15 ml. of physiologic saline. Hemolysis was complete

within 15 minutes. Aerobic incubation with N¹⁵-labeled glycine was then performed in the same manner as that described for the intact cell system.

To determine whether high oxygen tension was essential for significant synthesis of heme in this *in vitro* system, in Experiment 80-9 the incubation was performed with the flask open to air.

RESULTS AND DISCUSSION

Blood from normal rabbits with reticulocyte counts ranging from 0.0 to 5.9 per cent yielded hemin with concentrations of 0.006 to 0.036 atom per

Table I Blood from Normal Rabbits 25 ml. of blood were incubated in an atmosphere of 95 per cent O_2 -5 per cent O_2 -5 at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N^{15} .

Experiment No.	Reticulocytes	N ¹⁵ concentration in hemin
	per cent	alom per cent excess
39	0.8	0.011
70-1	1.4	0.015
75-1	2.0	0.006
75-2	3.5	0.011
75-3	1.7	0.016
73-2	2.1	0.014
73-1	0.0	0.029
84-1	5.9	0.023
84-2	4.0	0.036
84-3	2.5	0.032
85-2	1.7	0.018
85-3	1.8	0.023
85-4	1.9	0.020

cent excess N¹⁵ (Table I). Blood samples from rabbits in which reticulocytosis was induced by bleeding or phenylhydrazine administration, however, yielded hemin with much higher concentrations, 0.094 to 0.307 atom per cent excess N¹⁵ (Table II). Inasmuch as the glycine was labeled with 32 atom per cent excess N¹⁵, and it is reasonable to assume that the N¹⁵ concentration in the newly formed heme is of similar magnitude, the isotope values in the hemin (Table II) reveal that approximately 0.3 to 0.9 per cent of the total heme in the *in vitro* system was newly synthesized during the 24 hour period of incubation.

These findings demonstrate that the non-nucleated but immature mammalian erythrocyte is capable of synthesizing heme in vitro. This capacity for heme synthesis is present to a significant degree in rabbit blood characterized by an increased number of reticulated cells, whereas significant synthesis does not occur with normal mature erythrocytes.

The data in Table III indicate the effect of repeated bleeding on the reticulocyte counts and the capacity for heme synthesis. Elevated reticulocyte counts are associated with appreciable concentrations of N¹⁵ in hemin but no close correlation between the height of the reticulocyte count and the hemin isotope concentration exists. The findings in Experiments 75-1 and 75-3 (Table III) are of interest. Blood from normal rabbits with reticulocyte counts of 1.7 and 2.0 per cent yielded N¹⁵ values in hemin of

Table II
Rabbit Blood with Reticulocytosis

25 ml. of blood were incubated in an atmosphere of 95 per cent O_2 -5 per cent CO_2 at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N^{15} .

Experiment No.	Method of produc- ing reticulocytosis	Reticulocytes	N ¹⁵ concentra- tion in hemin	Remarks
		per cent	alom per cent excess	
33	Bleeding	35.0	0.178	
45	"	17.4	0.094	
5 8	"	15.1	0.236	
5 9	· "	8.2	0.182	
60	"	24.9	0.269	
62	"	8.6	0.140	
65	Acetylphen-	38.6	0.196	
	ylhydrazine			
67-2	"	39.0	0.148	
80-2	"	88.3	0.221	
80-9	"	67.3	0.272	Incubated in air
74	"	37.8	0.016	" " 95% N ₂ -5% CO ₂ atmosphere
80-3	"	88.7	0.307	Cells suspended in saline
72	"	23.0	0.002	Hemolyzed with saponin
80-5	"	61.6	0.004	" " "
93	"	82.6	0.007	" " distilled
				water

insignificant concentration. In Experiment 75-1, 2 days after the withdrawal of 25 ml. of blood, a N¹⁵ concentration in hemin of 0.056 atom per cent excess was achieved with a reticulocyte count of only 1.5 per cent, and in Experiment 75-3, 4 days after the first bleeding and 2 days after a second bleeding, with a reticulocyte count of 1.7 per cent the N¹⁵ concentration in hemin was 0.088 atom per cent excess. These isotope concentrations represent significant synthesis of heme. Two possible explanations of these results in the presence of normal reticulocyte counts may be offered. (a) The capacity of reticulocytes for synthesis of heme may vary widely. It

is reasonable to assume that this capacity is a function not only of the number of reticulocytes but of their age and morphologic development. (b) Synthesis of heme may be performed *in vitro* not only by reticulocytes but by other immature erythrocytes which, however, are not stained by brilliant cresyl blue.

Table III

Effect of Repeated Bleeding on Rabbits

25 ml. of blood were incubated at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N¹⁵. In Experiment 75-1 bleedings of 25 ml. each were made on 0, 2, 4, 7, and, 10 days; in Experiment 75-3 bleedings of 25 ml. were made on 0, 2, 4, and 7 days; in Experiment 85-2 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, 6, and 7 days; in Experiment 85-3 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, 6, and 7 days; in Experiment 85-4 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, and 6 days.

Experiment No.	Day	Reticulocytes	N ¹⁶ concentration ir hemin
		per cent	alom per cent excess
75-1	0	2.0	0.006
	2	1.5	0.056
	7	5.0	0.023
	10	19.5	0.096
75-3	0	1.7	0.016
	4	1.7	0.088
	7	10.0	0.177
85-2	0	1.7	0.018
	1	2.7	0.027
	6	20.0	0.125
	7	21.1	0.114
85-3	0	1.8	0.023
	1	3.3	0.029
	2	5.5	0.128
	4	13.8	0.109
	6	24.7	0.084
	7	25.1	0.152
85-4	0	1.9	0.020
	1	2.6	0.031
	2	3.8	0.033
	4	9.0	. 0.109
	6	23.7	0.057

It has been generally agreed that, although the reticulocyte is an immature erythrocyte, the deposition of hemoglobin is completed prior to denucleation (5, 6). The demonstration that the non-nucleated immature erythrocyte is capable of synthesizing heme *in vitro* raises the possibility that a significant, although perhaps small, proportion of heme formation

occurs in vivo in the non-nucleated immature cell in the bone marrow and in the peripheral blood.

To consider the *in vitro* synthesis of heme as evidence of *in vitro* hemoglobin formation, one must assume that the immature non-nucleated erythrocyte contains globin or is capable of synthesizing globin and that coupling of heme to globin can occur in the erythrocyte at this stage of its development. Evidence obtained in studies with duck erythrocytes suggests that globin can be synthesized *in vitro*. The finding of synthesis of heme and of the probable synthesis of hemoglobin by the non-nucleated immature cell represents a departure from the general belief that hemoglobin formation is completed prior to the disappearance of the nucleus from the red cell.

It appears, on the basis of the available evidence and on the assumptions which have been noted, that a significant but minor part of hemoglobin synthesis is carried out by the non-nucleated immature red cell. The ability of this cell to synthesize heme and, probably, hemoglobin is pertinent to the problem of the time relationships between hemoglobin deposition in the red cell and release of the red cell into the circulation. Earlier studies (7) in normal humans and in patients with hematologic disorders have shown that red blood cells containing N15-labeled heme appear in the peripheral blood within several hours after the oral administration of N¹⁵-labeled glycine. These studies suggest that the human erythrocyte is released into the circulation at a time which approximates the completion of hemoglobin deposition in the cell. If the red cell entering the circulation is mature, hemoglobin deposition is probably completed shortly before release of the cell from the bone marrow. This view is supported by the absence of significant heme synthesis in vitro by normal human blood (2). If, however, the red cell entering the circulation is immature, hemoglobin deposition may not yet be complete and further hemoglobin synthesis may occur in the peripheral blood.

In vitro synthesis of heme by reticulocytes offers an alternative explanation for increases in free erythrocyte protoporphyrin noted by Watson et al. (8) on incubation of human and rabbit blood samples containing immature erythrocytes. These increases were considered by these authors to be the result of intracorpuscular degradation of hemoglobin. It is possible that the increase in protoporphyrin represents at least in part an actual synthesis of new protoporphyrin in vitro.

No significant synthesis of heme occurred under the anaerobic conditions described (Experiment 74 in Table II) nor with the cells hemolyzed by saponin (Experiments 72 and 80-5) or distilled water (Experiment 93). Satisfactory synthesis of heme was observed on incubation of whole blood in air (Experiment 80-9).

¹ Shemin, D., London, I. M., and Rittenberg, D., J. Biol. Chem., 183, 757 (1950).

SUMMARY

The immature non-nucleated rabbit erythrocyte is capable of synthesizing heme *in vitro*.

We wish to acknowledge the valuable assistance of Miss Martha Yamasaki and to thank Mr. I. Sucher for the isotope analyses.

BIBLIOGRAPHY

- 1. London, I. M., Shemin, D., and Rittenberg, D., J. Clin. Invest., 28, 796 (1949).
- 2. London, I. M., Shemin, D., and Rittenberg, D., J. Biol. Chem., 173, 797 (1948).
- 3. Fischer, H., Org. Syntheses, 21, 53 (1941).
- Küster, W., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, 1, Teil 8, 208 (1922).
- 5. Wintrobe, M. M., Clinical hematology, Philadelphia (1946).
- 6. Thorell, B., Studies on the formation of cellular substances during blood cell production, London (1947).
- London, I. M., Shemin, D., West, R., and Rittenberg, D., J. Biol. Chem., 179, 463 (1949).
- 8. Watson, C. J., Grinstein, M., and Hawkinson, V., J. Clin. Invest., 23, 69 (1944).

THE SYNTHESIS OF PROTOPORPHYRIN IN VITRO BY RED BLOOD CELLS OF THE DUCK*

BY DAVID SHEMIN, IRVING M. LONDON, AND D. RITTENBERG

(From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, November 14, 1949)

Synthesis of heme has been shown to occur in vitro with the nucleated red blood cells of the duck (1). Incubation of duck blood with N¹⁵-labeled glycine results in the formation of N¹⁵-labeled heme. The in vitro system offers many biological and technical advantages for a study on the biosynthesis of porphyrins. The synthesis is rapid, the blood of birds is readily available, and large amounts can be obtained which permit comparative studies. The in vitro system, as compared to the whole animal, requires relatively small amounts of labeled compounds which are slightly diluted.

This communication will describe the use of the red blood cells of the duck as a system for the study of the biosynthesis of the porphyrin, heme.

EXPERIMENTAL

Incubation Experiments—Adult ducks were exsanguinated, without anesthesia, by cutting the exposed jugular veins and collecting the blood in a vessel containing 10 to 20 mg. of heparin. The heparinized blood was filtered through several layers of cheese-cloth to remove any débris, and about 3 mg. each of sodium benzyl penicillin and streptomycin calcium chloride complex were added to each 100 ml. of blood. Usually about 150 to 250 ml. of blood were obtained. Approximately 20 ml. of blood were used for each experiment. The effect of each factor or condition on heme synthesis was determined by comparison with the heme synthesis observed in a control blood sample. In most experiments the compounds were added without attempting to maintain isotonicity, while in some cases the substances were added as isotonic solutions. These conditions will be indicated in individual experiments. The glycine employed contained 32 atom per cent excess N¹⁵.

The blood samples were incubated with shaking at 37°. Usually little or no hemolysis occurred as judged by the appearance of the plasma following incubation. A few samples in which hemolysis had occurred were discarded.

Hemin Isolation—After incubation the blood samples were centrifuged,

* The work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

plasma was removed, and a volume of water equal to that of the red cells was added. The red cell suspension was homogenized for 5 to 10 minutes in a Waring blendor. The crude hemin, prepared by the usual method (2, 3) from 20 ml. of blood, was stirred with 0.5 ml. of pyridine. After 5 to 10 minutes 4 ml. of chloroform were added and the solution was filtered. 15 ml. of acetic acid, saturated with sodium chloride, were added to the filtrate and the mixture heated to 105°. 0.1 ml. of concentrated hydrochloric acid was then added and the hemin was allowed to crystallize at room temperature for 24 hours. The crystals were separated by centrifugation and washed successively with 50 per cent acetic acid, water, alcohol, and ether (3, 4). The hemin was analyzed for its isotopic content in the usual manner.

Synthesis of Heme from Glycine in Vitro—To each of four 20 ml. samples of whole duck blood, 200 mg. of isotopic glycine were added. The blood was incubated at 37° for 24 hours in an atmosphere of 95 per cent O₂-5 per cent CO₂. The hemin isolated was found to contain 0.126 atom per cent excess N¹⁵. This hemin was converted by the method of Grinstein (5) to protoporphyrin IX dimethyl ester and purified by chromatography and recrystallization. The N¹⁵ concentration of this compound, 0.124 atom per cent excess N¹⁵, was, within the experimental error, the same as for the hemin from which it was derived.

C₃₆H₃₆O₄N₄. Calculated, N 9.5; found, N 9.6

In another experiment five 30 ml. samples of whole blood, each containing 125 mg. of isotopic glycine, were incubated as above. The isotope concentrations of the pooled hemin, directly isolated and after each of two recrystallizations, were 0.068, 0.070, and 0.072 atom per cent excess N¹⁵ respectively. These differences lie within the experimental error of the analytical method.

Synthesis As Function of Time of Incubation—20 ml. samples of blood from the same duck were incubated with 10, 50, and 100 mg. of isotopic glycine for 6, 12, and 24 hours. The results shown in Fig. 1 demonstrate that the rate of synthesis of heme is most rapid during the first few hours; the rate then decreases but the synthesis continues for at least 24 hours.

N¹⁵ Concentration of Heme As Function of Amount of Glycine Added—To study the effect of glycine concentration on the synthesis, whole blood samples (16 to 24 ml.) were incubated with 1 to 300 mg. of isotopic glycine. Glycine was added either in crystalline form or as an isotonic solution. The data in Tables I and II show that the maximum N¹⁵ concentration in the heme is attained by the addition of about 1 mg. of glycine per ml. of blood. The amount of glycine required to yield a maximum N¹⁵ concentration in the hemin will depend clearly on the amount of non-

isotopic glycine present in the blood sample. The synthetic rate does not depend on whether the glycine is added as an isotonic solution or dissolved in the blood (compare Experiments VP-33 in Tables I and II).

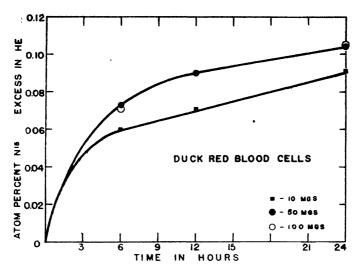


Fig. 1. Effect of concentration of glycine and time of incubation on N¹⁵ concentration in heme of duck erythrocytes.

Table I N^{15} Concentration in Heme As Function of Amount of Glycine Added Duck blood incubated for 24 hours with different amounts of isotopic glycine added in solid form.

N15 concentration in hemin

Glycine added	Experiment VP-28, 16 ml. blood	Experiment VP-33, 24 ml. blood	Experiment VP-36, 20 ml. blood
mg.	alom per ceni excess	alom per cent excess	alom per ceni excess
5	••	0.084	0.097
10	0.123	0.096	0.115
25	0.140	0.128	0.114
50	0.174	0.116	0.130
100	0.145	0.129	· 0.127
200	0.136	0.123	0.126
300		0.118	0.105

In one experiment, however, for some unexplained reason, the N¹⁵ concentration reached a maximum value on incubation with 3 mg. of glycine per ml. and then declined (see Experiment VP-28, Table I).

Influence of Temperature on Rate of Synthesis-To test the effect of tem-

perature on the rate of synthesis of hemin one 20 ml. sample of blood was incubated with 200 mg. of glycine at 37°, while a similar sample was kept at 5° for 24 hours. The hemin of the sample kept at 37° contained 0.066 atom per cent excess N¹⁵, while the hemin of the sample kept at 5° contained only 0.006 atom per cent excess N¹⁵.

Influence of Storage of Blood on Synthesis of Heme—Storage of blood at 0–5° does not lead to rapid deterioration of the system involved in the synthesis of heme. 25 ml. of a freshly drawn sample of whole blood were incubated at 37° for 24 hours with 100 mg. of isotopic glycine. The hemin isolated contained 0.123 atom per cent excess N¹⁵. Another sample of the same blood was kept for 48 hours in the refrigerator and then incubated with 100 mg. of isotopic glycine for 24 hours at 37°. The hemin

Table II

Nº18 Concentration in Heme As Function of Amount of Glycine Added

Duck blood incubated for 24 hours with different amounts of isotopic glycine added as an isotonic solution.

		N15 concentration in hemin		
Glycine added	Water added	Saline added	Experiment VP-21, 20 ml. blood	Experiment VP-33, 24 ml. blood
mg.	ml.	ml.	alom per cent excess	atom per cent excess
1	0.04	8.64	0.046	
5	0.22	8.46		
10	0.43	8.25	0.091	0.109
25	1.09	7.59		
50	2.17	6.51	0.104	0.123
100	4.34	4.34	0.105	0.129
200	8.68			0.122

isolated contained nearly the same isotope concentration, 0.118 atom per cent excess N¹⁵.

Blood from another duck was kept in the refrigerator for 1 week prior to incubation at 37° for 24 hours with 80 mg. of glycine. The hemin isolated contained 0.091 atom per cent excess N¹⁵.

In another experiment samples of blood were shaken at 37° for varying lengths of time (6 to 24 hours) prior to the addition of 50 mg. of isotopic glycine. Five 20 ml. samples of blood were treated as follows: Sample 1 was incubated with 50 mg. of glycine immediately after the bleeding; Samples 2, 3, 4, and 5 were shaken at 37° for 5, 10, 24, and 24 hours respectively. After these periods of time, 50 mg. of glycine were added to each sample and the samples were incubated an additional 24 hours. In Sample 5, 50 mg. of glucose were added together with the glycine. The

data shown in Table III indicate that storage at 37° impairs only slowly the synthetic mechanism.

Influence of Oxygen Tension—Hemin obtained from blood samples incubated in an atmosphere of 95 per cent O₂-5 per cent CO₂ contained the same concentration of N¹⁵ as hemin from blood samples incubated in air.

TABLE III

Effect on Synthesis of Heme on Incubation at 37° Prior to Addition of Isotopic

Glycine

20 ml. duck blood samples were incubated with 50 mg. of isotopic glycine for 24 hours after being shaken at 37° for varying lengths of time.

Time kept at 37° prior to incubation with glycine	N ¹⁵ concentration in hemin
hrs.	alom per cent excess
0	0.188
5	0.124
10	0.111
24	0.111
24*	0.106

^{*} With 50 mg. of glucose.

TABLE IV
Synthesis of Heme As Function of O₂ Tension

Samples of blood were incubated with isotopic glycine in an atmosphere of 95 per cent O₂-5 per cent CO₂, in air, or under anaerobic conditions. The samples were made anaerobic by passing a 95 per cent N₂-5 per cent CO₂ gas mixture through the flasks for different lengths of time and the flasks were then stoppered.

Experiment No. Anaerobic flasks, flushed with N2-CO2 95 per cent Or5 per Air cent CO2 10 min. 5 min. atom per cent excess atom per cent excess atom per cent excess atom per cent excess VP-8 0.1260.122VP-34 0.1330.0630.0430.1340.025VP-38 0.1140.030VP-23 0.0890.093

N15 concentration in hemin

However, hemin from blood which had been incubated in an atmosphere of 95 per cent N_2 -5 per cent CO_2 tontained considerably less N^{15} than that isolated from samples incubated under aerobic conditions (see Table IV).

Influence of Progressive Washing of Red Blood Cells—These experiments were carried out to determine whether any constituent of the plasma is required for the synthesis of heme in vitro.

The blood obtained from a duck was divided into six 20 ml. portions. One sample was left unchanged. The others were centrifuged and the plasma removed. The cells from one of these samples were resuspended in a mixture of 8 ml. of 0.9 per cent saline and 3 ml. of 0.154 m phosphate buffer, pH 7.4. The cells from the others were washed once or more with equal volumes of 0.9 per cent saline and similarly suspended in phosphate buffer or in the original plasma. Each of the six samples was then incubated with 50 mg. of isotopic glycine for 24 hours in air. The results are given in Table V.

Synthesis with Cell-Free Preparations—Two 20 ml. samples of blood were frozen with solid CO₂ and allowed to thaw. Microscopic examination indicated that complete hemolysis had occurred. These preparations

TABLE V

Influence of Washing of Red Blood Cells on Synthesis of Heme

Cells from 20 ml. of blood samples were washed one or more times, resuspended either in phosphate buffer (pH 7.4) or in the original plasma, and incubated for 24 hours with 50 mg. of glycine.

Blood samples		N ¹⁵ concentration in hemir
No. of washings	Resuspended in	
		alom per cent excess
Control (whole blood)		0.113
0	Buffer	0.103
1	44	0.093
3	cc .	0.084
5	"	0.081
5	Plasma	0.120

were incubated with 50 mg. of isotopic glycine for 24 hours at 37°. The hemin isolated contained 0.001 and 0.006 atom per cent excess N¹⁵. Hemin from a control sample contained 0.114 atom per cent excess N¹⁵.

In another experiment hemin isolated from blood which had been homogenized with an equal volume of water prior to incubation contained 0.005 atom per cent excess N¹⁵; hemin of the control contained 0.130 atom per cent excess N¹⁵.

An acetone powder was prepared from 13 ml. of washed red blood cells in the usual manner. This powder (5 gm.) was dissolved in 0.10 m phosphate buffer, pH 7.4, and incubated with 200 mg. of isotopic glycine, 50 mg. of sodium acetate, 200 mg. of adenosine triphosphate, and 3 mg. of pyridoxal hydrochloride. The hemin isolated contained 0.004 atom per cent excess N¹⁶.

Cyanide Inhibition-Six 24 ml. samples of blood from a duck were incu-

bated with 50 mg. of N¹⁵-glycine and varying amounts of cyanide for 24 hours in air. The results are shown in Table VI.

Peptide Bond Synthesis—A 25 ml. sample of blood was incubated under a 95 per cent O₂-5 per cent CO₂ atmosphere for 24 hours with 10 mg. of histidine monohydrochloride containing 69 atom per cent excess N¹⁵ in one of the imidazole nitrogen atoms (6). After the incubation period, 1 gm. of non-isotopic histidine was added and the mixture was dialyzed. Dialysis was continued for several days after the dialysate gave a negative test for histidine. The crude hemoglobin fraction was hydrolyzed with 20 per cent hydrochloric acid. Histidine was isolated from the hydrolysate (7) and contained 0.048 atom per cent excess N¹⁵.

TABLE VI $Cyanide\ Inhibition$

24 ml. samples of duck blood were incubated with 50 mg. of glycine for 24 hours with different concentrations of cyanide added as an isotonic solution.

Sample No.	Cyanide	N15 concentration in hemin
	¥	alom per cent excess
1, 2	0	0.133, 0.134
3	10^{-2}	0.019
4	10-8	0.082
5	10-4	0.130
6	10-5	0.137

Purine Synthesis—Four 40 ml. samples of blood were incubated in air for 24 hours with 200 mg. of isotopic (32 atom per cent excess N¹⁵) glycine in each. The plasma was removed by centrifugation and the cells were washed with isotonic saline and dried by lyophilization. The purines were isolated as the copper derivatives by the method of Graff and Maculla (8) and guanine was isolated from the copper purines by isoelectric precipitation. The N¹⁵ concentration of the guanine was 0.012 atom per cent excess N¹⁵.

DISCUSSION

In the normal mammal the heme of the mature, circulating red blood cell is not in the dynamic state and remains within the cell until the erythrocyte disintegrates (9). The appearance of labeled heme in the red blood cell of the mammal after the feeding of isotopic glycine results from the synthesis of heme in the red blood cell while it is still in the bone marrow. In the bone marrow the precursors of the mature, circulating blood cells are nucleated. As the circulating red blood cells in the duck are nucleated, they are tested for their ability to synthesize heme in vitro.

This was found to occur. It should be noted, however, that the nucleus is not essential, for synthesis can occur with mammalian non-nucleated but immature erythrocytes (10). The erythrocytes of the peripheral blood of the duck, which are responsible for heme synthesis in vitro, may be immature cells. It is known that in ducks reticulocyte counts up to 20 per cent are a normal finding. It is as yet unknown whether all the nucleated cells, or only the immature nucleated red cells, are capable of carrying out synthesis of heme in vitro.

The rate of synthesis is maximum initially and falls off with time. Although the synthetic capacity of the cells is unimpaired at 5°, cells stored at 37° lose some of this ability in the first few hours but only slowly thereafter. The loss of synthetic capacity of the cells may be due either to the disappearance of a substance essential for heme synthesis or to cellular maturation.

Since, as has been demonstrated, glycine nitrogen is the source of all 4 nitrogen atoms of heme (11, 12), the amount of synthesis in these *in vitro* experiments is approximately 0.4 per cent $(0.13/32 \times 100)$ during the 1st day.

Even thoroughly washed cells can carry out the synthesis. Since it is known that acetic acid is required for the synthesis of heme (13), the washed cells must retain acetate or potential sources of acetate.

Anaerobiosis inhibits the reaction. Appreciable inhibition by cyanide requires concentrations of 10^{-3} M or greater. Under the conditions of the experiments described here destruction of the organization of the red cell abolishes the synthesis.

It should be noted that the red cell of the duck can incorporate histidine into the cell proteins, *i.e.* synthesize proteins. By isolation and isotope analysis of the histidine of the proteins, it is possible to estimate the fraction of the proteins formed during the 24 hour incubation. Since the isolated histidine had an isotope concentration about 1/480, *i.e.* 0.048/23.0 of that added, at least 0.21 per cent of the histidine in the protein has been incorporated during the incubation period. This is similar to the rate of porphyrin synthesis in these experiments. This suggests that globin, as well heme as, is made as required for hemoglobin synthesis.

SUMMARY

- 1. The synthesis of heme from glycine was demonstrated in the nucleated red blood cell of the duck in vitro.
- 2. The synthesis of peptide bonds in this in vitro system was also demonstrated.
- 3. The conditions under which heme is synthesized in vitro in the nucleated red blood cell of the duck are described.

We wish to acknowledge the valuable assistance of Miss Selma Friedman and we wish to thank Mr. Irving Sucher for the isotope analyses.

BIBLIOGRAPHY

- 1. Shemin, D., London, I. M., and Rittenberg, D., J. Biol. Chem., 173, 799 (1948).
- 2. Nencki, M., and Zaleski, J., Z. physiol. Chem., 30, 384 (1900).
- 3. Fischer, H., Org. Syntheses, 21, 53 (1941).
- Kuster, W., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, 1, Teil 8, 201 (1922).
- 5. Grinstein, M., J. Biol. Chem., 167, 515 (1947).
- 6. Tesar, C., and Rittenberg, D., J. Biol. Chem., 170, 35 (1947).
- 7. Foster, G. L., and Shemin, D., Org. Syntheses, 18, 43 (1938).
- 8. Graff, S., and Maculla, A., J. Biol. Chem., 110, 71 (1935).
- 9. Shemin, D., and Rittenberg, D., J. Biol. Chem., 166, 627 (1946).
- London, I. M., Shemin, D., and Rittenberg, D., J. Biol. Chem., 173, 797 (1948);
 183, 749 (1950).
- 11. Wittenberg, J., and Shemin, D., J. Biol. Chem., 178, 47 (1949).
- 12. Muir, H. M., and Neuberger, A., Biochem. J., 45, 163 (1949).
- 13. Bloch, K., and Rittenberg, D, J. Biol. Chem., 159, 45 (1945).

THE RELATIONSHIP OF SERINE TO PORPHYRIN SYNTHESIS*

BY DAVID SHEMIN, IRVING M. LONDON, AND D. RITTENBERG

(From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, November 14, 1949)

The specific utilization of glycine for the biosynthesis of protoporphyrin has been demonstrated (1, 2). Proline, glutamic acid, leucine, and ammonia are not directly utilized for such synthesis (2). We now find that the administration to rats of N¹⁵-labeled serine or acetylglycine results in isotope concentrations of the hemin similar to those obtained following

• Compound fed	N15 concentration	N15 concentration in hemin	N ¹⁵ Concentration in hemin		
		Found			
	alom per cent excess	alom per cent excess	atom per cent excess		
Glycine	32	0.442	1.37		
"	32	0.451	1.41		
Acetylglycine	32	0.427	1.34		
"	32	0.374	1.17		
DL-Serine .	11.6	0.155	1.33		
"	11.6	0.164	1.41		

^{*} Calculated on the basis that the compound fed contained 100 per cent excess N^{15} .

labeled glycine administration (Table I). It is probable that prior to utilization the acetylglycine is hydrolyzed to glycine. This hypothesis finds support in the experiments in which acetylglycine and glycine were incubated with duck blood (Table II). Little incorporation of labeled nitrogen was found after incubation with acetylglycine, though appreciable synthesis took place with glycine itself. It is therefore improbable that acetylglycine is an intermediate in the formation of heme. Presumably the duck erythrocytes, in contrast to the whole rat, are incapable of hydrolyzing added acetylglycine.

The data in Table I show that in the rat serine, as well as glycine, can serve as a precursor of heme. It is known that in the mammalian organism serine is rapidly converted to glycine with retention of the amino

^{*} The work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

Table II

Comparison of N¹⁵ Concentration in Heme after Incubating Duck Blood with

Isotopic Compounds

20 ml. of whole duck blood were incubated for 24 hours at 37° with N¹⁵-labeled compounds. All the compounds were added as isotonic neutral solutions.

VP-19-1 VP-19-2 VP-19-3 VP-19-4 VP-23-1 VP-23-2 VP-23-3 VP-23-4	Compou <u>n</u> d added	N15 concentra-	N ¹⁵ concentra- tion in hemin	N ¹⁵ concentra- tion in hemin Calculated*	
	•	11011	Found		
		atom per cent excess	alom per cent excess	atom per cent excess	
VP-19-1	Glygine (0.33 mm)	32	0.135	0.42	
VP-19-2	" (0.67 ")	32	0.199	0.62	
VP-19-3	" (1.33 ")	32	0.139	0.44	
VP-19-4	DL-Serine (1.0 mm)	11.6	0.048	0.41	
VP-23-1	Glycine (0.25 mm)	32	0.090	0.28	
VP-23-2	DL-Serine (0.5 mm)	32	0 040	0.12	
VP-23-3	NH ₄ Cl (0.25 mm)	32	0.010	0.03	
VP-23-4	L-Aspartic acid (0.25 mm)	31.3	0.010	0.03	
VP-23-6	DL-Alanine (0 5 mm)	23	0.004	0.02	
VP-23-7	Acetylglycine (0.25 mm)	32	0.012	0.04	

^{*} Calculated on the basis that the compound added contained 100 per cent excess N¹⁶.

Table III

Nºº Concentration in Heme of Duck Blood after Incubation with Non-Isotopic Amino
Acids and Labeled Glycine or Serine

Experiment No.	Isotopic compound	N ¹⁵ con centra- tion	Non-isotopic compound	N ¹⁸ concentra- tion in hemin	N ¹⁵ concentra- tion in hemin ————————————————————————————————————
		aiom per ceni excess		atom per cent excess	aiom per cent excess
VP-23-1	Glycine (0.25 mm)	32		0.090	0.28
VP-23-2	" (0.25 ")	32	DL-Serine (2.75 mm)	0.076	0.24
VP-23-3	DL-Serine (0.5 mм)	32		0.040	0.13
VP-23-4	" (0.5 ")	32	Glycine (2.5 mм)	0.005	0.02
VP-20-1	Glycine (0.25 mм)	32	L-Serine (2 mm)	0.133	0.42
VP-20-2	" (0.25 ")	32	L-Alanine (2 mм)	0.133	0.42
VP-20-3	рь-Serine (1.0 mм)	11.6	Glycine (1.75 mm)	0.011	0.09
VP-20-4	" (1.0 ")	11.6	Alanine (1.6 mm)	0.023	0.20

^{*} Calculated on the basis that the labeled compound added contained 100 per cent excess N^{16} .

group (3). Serine may therefore function as a precursor of heme by virtue of its conversion to glycine. It is also known that glycine can be

converted to serine (4-6). Either of the reactions shown below could explain the experimental results.

Serine
$$\rightarrow$$
 glycine \rightarrow heme (1)
Glycine \rightarrow serine \rightarrow heme (2)

In order to evaluate these possibilities, samples of duck blood were incubated with either isotopic glycine or isotopic serine. In both cases isotopic hemin resulted (Table II). However, when the isotopic serine was mixed with large amounts of non-isotopic glycine, the isotope concentration in the hemin was extremely low (Table III). On the other hand, when isotopic glycine was incubated with large amounts of non-isotopic serine, hemin of high N¹⁵ concentration was obtained. These results demonstrate that serine is utilized for porphyrin synthesis by virtue of its conversion to glycine (Reaction 1).

We have tested synthesis of heme with other nitrogenous compounds in duck blood. Negative results were obtained for aspartic acid, alanine, and ammonia (Table II).

EXPERIMENTAL

The N^{15} -labeled amino acids were prepared by methods previously described (3, 7).

The experimental procedures were similar to those previously described (2, 8). In incubation experiments the amino acid hydrochlorides were neutralized and added as isotonic solutions.

SUMMARY

- 1. Serine is utilized for the synthesis of heme by virtue of its conversion to glycine.
 - 2. Acetylglycine as such is not utilized for synthesis of heme.

We wish to acknowledge the valuable assistance of Miss Selma Friedman and we wish to thank Mr. I. Sucher for the isotope analyses.

BIBLIOGRAPHY

- 1. Shemin, D., and Rittenberg, D., J. Biol. Chem., 166, 627 (1946).
- 2. Shemin, D., and Rittenberg, D., J. Biol. Chem., 166, 621 (1946).
- 3. Shemin, D., J. Biol. Chem., 162, 297 (1946).
- Ehrensvärd, G., Sperber, E., Saluste, E., Reio, L., and Stjernholm, R., J. Biol. Chem., 169, 759 (1947).
- Winnick, T., Moring-Claesson, I., and Greenberg, D. M., J. Biol. Chem., 175, 127 (1948).
- 6. Sakami, W., J. Biol. Chem., 176, 995 (1948).
- 7. Schoenheimer, R., and Ratner, S., J. Biol. Chem., 127, 301 (1939).
- 8. Shemin, D., London, I. M., and Rittenberg, D., J. Biol. Chem., 183, 757 (1950).

ACETIC ACID METABOLISM IN TORULOPSIS UTILIS

I. THE CULTIVATION OF TORULOPSIS YEAST ON C'*H,C'*OOH AS THE SINGLE CARBON SOURCE

By J. BADDILEY, G. EHRENSVÄRD, R. JOHANSSON, L. REIO, E. SALUSTE, AND R. STJERNHOLM

(From the Wenner-Gren Institute of Experimental Biology and the Institute of Physics, University of Stockholm, Stockholm, Sweden)

(Received for publication, May 10, 1949)

During a series of experiments with labeled amino acids as single carbon substrates for the cultivation of *Torulopsis* yeast (1, 2), it was found necessary to make a thorough investigation of the intermediary metabolism of acetic acid in order to establish the main pathways of transformation of acetyl residues into amino acid structures. With this end in view, we have prepared acetic acid, labeled with C¹⁸ in the methyl group and C¹⁴ in the carboxyl, and have used this acid as a single carbon substrate for the cultivation of *Torulopsis* yeast, previously adapted to acetic acid. After growing for a certain time on this substrate, the yeast was isolated and subjected to appropriate treatments in order to separate soluble carbohydrates, fats, and proteins. The latter were then hydrolyzed and amino acids were isolated and degraded in order to localize and estimate the isotope content of different carbon atoms of the structures.

Preparation of C¹³H₃C¹⁴OOH—81 gm. of KC¹³N, with about 5 per cent excess C¹³ (Eastman Kodak), were precipitated as zinc cyanide and the latter mixed (after drying) with an equal weight of copper oxide and burned with oxygen in a quartz tube in 2 gm. portions. The carbon dioxide was absorbed in barium hydroxide and the barium carbonate was centrifuged, washed, and dried. Yield, 197 gm. of barium carbonate with 4.5 per cent excess C¹³.

Carbon dioxide, liberated from the carbonate by 30 per cent perchloric acid, was reduced to methanol according to the method of Nystrom, Yanko, and Brown (3). Our yield of methanol from 197 gm. of barium carbonate was 32 gm. of redistilled product, corresponding to 90 per cent. Methyl iodide was then prepared from the methanol by a modification of the method of Tolbert (4) and carbonated in a Grignard reaction with carbon dioxide containing about 5 mc. of C¹⁴. Isolation of potassium acetate was carried out according to Anker (5). The dry potassium acetate (about 50 gm.) was mixed carefully with concentrated sulfuric acid in a distilling flask and the concentrated acetic acid was distilled off at 112–119°. Yield, 23 gm. of 94 per cent C¹⁸H₃C¹⁴OOH with 3 to 4 per cent excess C¹³ in the methyl group, and about 3 mc. of C¹⁴ in the carboxyl group.

Experimental Conditions for Adaptation of Torulopsis Yeast to Acetic Acid—During the stages of adaptation of the yeast to acetate, we followed, in principle, the directions given by Roine (6) and Sperber (7) for small scale cultures. A few cells of a pure culture of Torulopsis utilis were cultivated under aerobic conditions in an ordinary wash-bottle, sterile air being passed through the culture at 30°. The medium consisted of 50 gm. of pure sucrose, 4 gm. of ammonium sulfate, 3 gm. of ammonium phosphate (secondary), 1.5 gm. of potassium sulfate, 1 gm. of magnesium sulfate, and 0.5 gm. of calcium chloride in 1000 ml. of tap water, of which 150 ml. were used during the first stage of cultivation. After the yeast had increased in wet weight to about 10 gm. (24 hours), it was transferred to a 1.2 liter Kluyver flask (see Sperber (7), Fig. 1, p. 12) containing 1000 ml. of the above medium and cultivated for 24 hours under vigorous aera-The pH was kept at 4.0 to 4.5, usually adjusted by addition of 1 N KOH. When the wet weight had reached 80 gm., the yeast was separated from the medium by centrifuging and suspended in 2000 ml. of the same medium in a 20 liter stainless steel Kluyver flask. During all cultivation procedures, foaming was kept under control by adding, from time to time, a few drops of Turkey-red oil. The culture in the 20 liter flask was permitted to grow during 24 hours with aeration corresponding to 20 liters of air per minute. At the end of this period (72 hours from the start) the yeast had consumed 100 gm. of sucrose and had increased its wet weight to about 250 gm. The medium was then diluted with tap water to 7000 ml., 50 ml. of ethyl alcohol and 10 gm. of ammonium sulfate were added, and cultivation was continued during 8 hours with intermittent addition of ethyl alcohol, 175 gm. in total. The volume was now 8000 ml.

At this stage, 1 gm. of acetic acid (partially neutralized with ammonia), together with 15 gm. of ethyl alcohol, was added to 4200 ml. of the yeast culture. The proportion of acetic acid subsequently was gradually increased and the alcohol was decreased. At 114 hours 102 gm. of alcohol had been added and 66 gm. of acetic acid. The volume of the culture was 7300 ml. and it contained approximately 450 gm. of yeast, which metabolized 2.5 gm. of acetic acid per hour. 2500 ml. of this culture were then diluted to 15,000 ml. with tap water and 12 gm. of acetic acid and 3 gm. of alcohol were added. In subsequent additions the amount of alcohol was further reduced until only acetate was being added. At 117 hours the volume of the culture was 16,000 ml. and there were approximately 260 gm. of yeast which utilized 7.0 gm. of acetic acid per hour at a steady rate.

During this adaptation to acetate the pH was maintained at 5.1 to 6.3 and the morphological appearance of the yeast cells was observed half hourly. If the budding rate decreased below 30 per cent, the adaptation had to be repeated from the ethanol stage. The disappearance of acetic

acid from the medium was followed by withdrawing 100 ml. samples of the suspension periodically, centrifuging off the yeast, distilling the cell-free solution with steam during 45 minutes, and, finally, titrating the acetic acid in the distillate.

At the 117 hour point isotopic acid (20 gm.) was added (half neutralized with ammonia) and a 100 ml. sample of the suspension was taken after 2 minutes in order to estimate the total amount of acetic acid, labeled plus non-labeled, present in the medium. This acid represents the starting material of the actual isotopic experiment. Further samples were taken at 60 and 120 minutes, the experiment being stopped after 180 minutes; i.e., in all, after 120 hours. The yeast was then killed by adding 20 ml. of 30 per cent perchloric acid, suspended carefully in water, and centrifuged. After washing again with water, centrifuging, and drying by treatment four times with 1000 ml. portions of absolute ethanol and then three times with 400 ml. of ether, the yeast had a dry weight of 82.5 gm.

Isotope Content of Yeast and Respiratory CO₂—During the later stages of the experiment the large Kluyver flask was provided with a close fitting top with three holes, one fitted with a separatory funnel for adding substrate, one with a wide glass tube leading to the bottom of the flask, enabling samples to be withdrawn at intervals, and the third with an outlet nozzle for connection to a series of CO₂ absorption flasks containing Ba-(OH)₂ solution. Four of the absorption flasks were coupled in series, and at intervals the flasks were exchanged against another absorption unit of the same type. Each flask contained about 1000 ml. of saturated baryta solution. In this way nine different samples of respiratory CO₂ were collected, representing the total output of CO₂ during the last 3 hours of the experiment. C¹³ and C¹⁴ determinations of the isolated barium carbonate are shown in Table I, the last columns of which give the percentage of the methyl and the carboxyl groups of the acetic acid in the medium appearing as respiratory CO₂.

The latter series of figures is based upon the isotope content of the acetic acid of the medium taken 2 minutes after addition of the isotopic acetic acid. The acetate was isolated as its silver salt by steam distillation of the sample, neutralization with KOH, evaporation to a small volume, and precipitation with silver nitrate solution. The silver acetate was burned to CO₂, which was isolated as barium carbonate and analyzed for C¹³ and C¹⁴. The former is expressed in atom per cent excess, the latter in counts per minute per 15 mg. of BaCO₃ sample under standard conditions. All isotope measurements were made in the same way. The figures thus obtained for the isotopic acetate actually present at 2 minutes after the start of the experiment were 1.21 per cent C¹³ excess and 4925 c.p.m., representing

2.42 per cent C¹³ in the CH₃, and 9850 c.p.m. in COOH. With these values taken to unity, the ratio 'C¹⁴:'C¹³, calculated for the respiratory CO₂, as shown in Table I, remained at approximately 2 during the main part of the experiment, indicating that, for each methyl group of the acetic acid metabolized to CO₂ by the yeast, two carboxyl groups are involved in decarboxylation reactions. Assuming the validity of the Krebs cycle in Torulopsis, this value of 2 would reflect an analogous ratio of C¹⁴:C¹³ in the α-carboxyl group of glutamic acid and both carboxyls of aspartic acid at the end of the experiment. Preliminary determination of these ratios gave the figures 1.72 and 1.78, respectively, which agreed with the ratio

TABLE I
Isotope Content of Respiratory CO₂ Taken at Intervals

C¹³ in atom per cent excess, C¹⁴ counts per minute per 15 mg. of barium carbonate. 'C¹³ and 'C¹⁴ denote isotope content of respiratory CO₂ in per cent of the corresponding C¹³ content of the methyl group and C¹⁴ of the carboxyl group of the acetic acid present at the start of the experiment (2.42 per cent excess C¹³ and 9850 c.p.m. per 15 mg. of barium carbonate).

Time intervals	C18	C14	.C₁₁	,C₁4	'C18 + 'C14	,Си:,Си
min.						
0-2	0.02	418	0.8	4.2	5.0	5
2- 7	0.17	784	6.9	7.9	14.8	
7- 22	0.24	2920	10.0	29.9	39.5	2.99
22- 42	0.45	4655	18.5	47.0	65.5	2.55
42- 65	0.52	5155	21.4	52.2	73.6	2.45
65- 95	0.62	5185	25.6	52.3	77.9	2.04
95-125	0.65	5360	27.1	54.3	81.4	2.00
125-155	0.73	5915	30.0	59.8	89.8	1.99
155-180	0.76	4990	31.4	50.6	82.2	1.62

1.74 for the respiratory CO₂ during the last 60 minutes of the experiment (Table I).

Preliminary information about the isotope content of the samples of yeast taken at 2, 60, 120, and 180 minutes was obtained by combustion of a small amount of each of these samples to CO₂ and subsequent determination of C¹³ and C¹⁴. These values, 0.060, 0.123, 0.169, 0.180, and 29, 69, 305, and 348 c.p.m., show that, even at 60 minutes, the yeast had incorporated a considerable amount of isotopic carbon from the acetic acid. In order to investigate the partition of C¹³ and C¹⁴ in the three main amino acid groups, the acidic, neutral, and basic fractions, the yeast was hydrolyzed with 20 per cent HCl, evaporated to dryness, and electrodialyzed according to the method of Sperber (8). From the different fractions, total carbon was determined by combustion and amino acid

BADDILEY, EHRENSVÄRD, JOHANSSON, REIO, SALUSTE, AND STJERNHOLM

carboxyl liberated as CO₂ by the method of Van Slyke *et al.* (9). Table II gives the values of C¹³ and C¹⁴ for the total and the carboxyl C of each fraction. The total C values of the neutral fractions should be regarded with some reservation on account of their probable carbohydrate content. In the case of the basic fraction, paper chromatography showed a minor amount of ethanolamine and glucosamine.

The general picture of the incorporation of acetic acid into the three amino acid fractions, as shown in Table II, gives the impression that glutamic and aspartic acids have incorporated the isotopic carbon from

Table II

Isotope Content of Different Amino Acid Fractions Prepared from Four Samples
of Yeast Taken at Different Stages during Experiment

C18 in atom per cent excess, C14 counts per minute per 15 mg. of barium carbonate.

			Carbo	oxyl			Total					
Tinfe	Acid fraction Neutra			Basic fraction		Acid fraction		Neutral fraction		Basic fraction		
	C13	C14	C18	C14	Crs	C14	Cn	C14	C18	C14	Cra	C14
min.				}								
2	0.00		0.00		0.00		0.03		0.02		0.02	
	1	65		16		12		47		7]	24
60	0.08		0.02		0.01		0.16		0.07		0.08	
		524		249		102		231		54		108
120	0.15	İ	0.04		0.04		0.30		0.14		0.12	
		988		464		269		380		176		156
180	0.15		0.09		0.04		0.28		0.21		0.17	
		902		660		314		392		244		226

acetate at a higher rate than the other amino acids. This is by no means unexpected in view of the results obtained by Lifson et al. (10). The decrease of the isotopic content in the acidic fraction during the last hour of the experiment might imply that part of this fraction is utilized for the synthesis of other amino acids, the members of the basic fraction in general being formed more slowly than the neutral amino acids. A feature of definite interest is, however, the high C¹⁴ content in the neutral and basic fraction in comparison with the corresponding values of the carboxyls from these fractions. This means that a considerable amount of the carboxyl part of the acetic acid has become incorporated into the non-carboxylic parts of some amino acid or acids, both neutral and basic. In order to locate the actual sites of C¹⁸ and C¹⁴ derived from the acetic acid in the framework of each amino acid, the 180 minute fractions, corre-

sponding to about 40 gm. of protein as amino acids, were taken as starting material for isolation of individual amino acids in a pure state, each amino acid being subjected to degradation procedures in order to locate the C¹³ and C¹⁴ in the molecule. This work, the results of which are to be described in subsequent publications, will give some detailed information as to the function of acetic acid in the formation of individual amino acids.

SUMMARY

- 1. A detailed scheme is given for the cultivation of the yeast *Torulopsis utilis* on acetic acid and ammonia.
- 2. In a cultivation experiment, acetic acid, labeled with C¹³ in the methyl group and C¹⁴ in the carboxyl group, was used as a single carbon substrate for the growth of the yeast.
- 3. Isotope determinations of the respiratory carbon dioxide liberated during the experiment show a 'C¹⁴:'C¹³ ratio of about 2, in comparison to the corresponding ratio of the carbon atoms of acetic acid of the substrate, taken as 1, indicating that, for two carboxyl groups liberated from the acetic acid as CO₂, one methyl group is entering the cycle of metabolic breakdown, finally appearing as respiratory CO₂.
- 4. In agreement with this, the C¹⁸ content of the yeast substance formed is relatively higher than the corresponding C¹⁴ content, the latter appearing mainly in the carboxyls of the amino acids formed but also to some extent in the non-carboxylic parts of some neutral and basic amino acids.

Our thanks are due to the Swedish State Scientific and Medical Councils for financial support of this work.

BIBLIOGRAPHY

- Ehrensvärd, G., Sperber, E., Saluste, E., Reio, L, and Stjernholm, R., J. Biol. Chem., 169, 759 (1947).
- Ehrensvärd, G., Hogstrom, G., Reio, L., Sperber, E., and Saluste, E., Cold Spring Harbor Symposia Quant. Biol., 13, 81 (1948).
- Nystrom, R. F., Yanko, W. H., and Brown, W. G., J. Am. Chem. Soc., 70, 441 (1948).
- 4. Tolbert, B. M., J. Am. Chem. Soc., 69, 1529 (1947).
- Anker, H. S., J. Biol. Chem., 176, 1333 (1948).
- 6. Roine, P., Dissertation, University of Helsinki (1947).
- 7. Sperber, E., Ark. Kemi, Mineral. o. Geol., 21 A, No. 3, 116, 117 (1945).
- 8. Sperber, E., J. Biol. Chem., 166, 75 (1946).
- Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., J. Biol. Chem., 150, 251 (1943).
- Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., J. Biol. Chem., 176, 1263
 (1948).

ACETIC ACID METABOLISM IN TORULOPSIS UTILIS

II. METABOLIC CONNECTION BETWEEN ACETIC ACID AND TYROSINE AND A METHOD OF DEGRADATION OF THE PHENOLIC RING STRUCTURE IN TYROSINE

By J. BADDILEY, G. EHRENSVÄRD, E. KLEIN, L. REIO, AND E. SALUSTE

(From the Wenner-Gren Institute of Experimental Biology, University of Stockholm, Stockholm, Sweden)

(Received for publication, May 10, 1949)

A previous paper in this series (1) describes conditions necessary for the growth of *Torulopsis* yeast when acetic acid containing excess C¹³ in the methyl and C¹⁴ in the carboxyl group is fed as the sole source of carbon. This communication embodies a detailed study of the distribution of C¹³ and C¹⁴ in the tyrosine isolated from a hydrolysate of protein from this yeast.

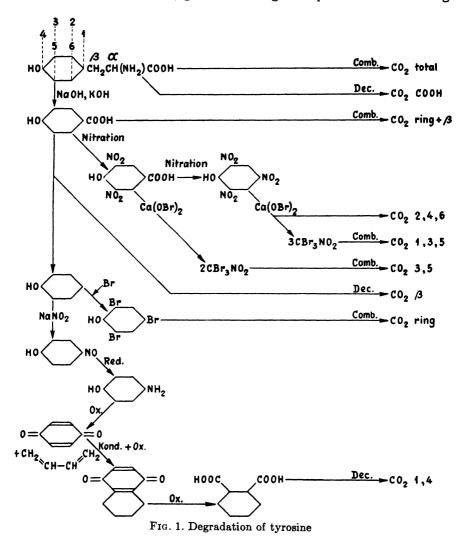
The neutral fraction of the protein hydrolysate was evaporated to a small volume, the pH adjusted to 5.8, and the temperature kept at 0° for 2 days. Tyrosine crystallized out (1006 mg. of crude product) and was recrystallized from water. 580 mg. of pure amino acid were obtained from 80 gm. of dry yeast. The preparation was shown by paper chromatography to contain no other amino acids.

Degradation Procedure—In order to obtain a complete picture of the isotope distribution in tyrosine, numerical values for the C^{13} and C^{14} concentrations in the following carbon atoms must be obtained: The carboxyl, the α , and the β atoms of the side chain, and finally the atoms of positions 1, 4, 3 and 5, and 2 and 6 of the benzene ring. Positions 3 and 5, like 2 and 6, are naturally indistinguishable. The scheme presented in Fig. 1 gives the general outline of the procedure.

All measurement of isotope concentration was carried out on barium carbonate samples derived from the carbon atoms from one or several different positions of the tyrosine molecule. The isotope concentration of the barium carbonate preparations will in the following calculation be designated as $C_{carboxyl}$, C_{α} , C_{β} , C_{1} , $C_{1,4}$, $C_{1,3,5}$, etc., the suffixes indicating the original position of the carbon atoms. Two or more suffixes, e.g. $C_{1,3,5}$, indicate that the measured isotope concentration is the mean value of the isotope concentrations of each of the atoms in the positions indicated.

Carboxyl carbon was determined readily by treatment with ninhydrin according to the method of Van Slyke et al. (2), the evolved carbon dioxide being collected and analyzed as barium carbonate. The value for C_{α}

was obtained indirectly by conversion of the main part of the tyrosine to 4-hydroxybenzoic acid by alkali fusion. A sample of this acid, on combustion to carbon dioxide, gave the average isotope content of the ring



atoms plus C_{β} , which, together with the average for the whole tyrosine molecule obtained by total combustion, made it possible to calculate the value for C_{α} by difference, from the carboxyl figure:

$$C_{\alpha} = 9C_{\text{total}} - 7C_{\text{hydroxybensoic acid}} - C_{\text{carboxyl}}$$

The carbon dioxide, evolved on heating a sample of this 4-hydroxybenzoic acid in quinoline at 250°, represented the β -carbon atom of tyrosine. while the other product of this reaction, phenol, isolated as 1,3,5-tribromophenol, on combustion to carbon dioxide gave the average of the 6-ring atoms. Nitration of a further part of the 4-hydroxybenzoic acid to 3,5dinitro-4-hydroxybenzoic acid, followed by treatment with calcium hypobromite at 40°, caused disruption of the ring, liberating carbon atoms 3 and 5 as bromopicrin (tribromonitromethane). The latter was isolated and burned to carbon dioxide, the isotope content of which gave values for C_{3.5}. Further vigorous nitration, with decarboxylation of the remaining 3,5-dinitro-4-hydroxybenzoic acid, produced pieric acid which, in turn, was converted to bromopic with calcium hypobromite. bustion of this compound gave carbon dioxide, representing atoms 1 and 3.5. The isotope content of 1 could then be obtained from $C_1 = 3C_{1.3.5}$ - 2C_{3,5}. Thus values for C₁ and C_{3,5} were obtained, while the difference between the carbon of the phenol and $C_{1,3,5}$ gave $C_{2,4,6}$. In order to distinguish between C4 and C2,6, the main part of the phenol obtained by decarboxylation of 4-hydroxybenzoic acid was converted to 4-nitrosophenol by treatment with nitrous acid. This, on reduction to 4-aminophenol, followed by oxidation, gave p-benzoquinone, which was condensed with 1,3-butadiene to dihydronaphthoquinone. Oxidation of the latter by the method of Fieser (3) gave naphthoquinone, which could be oxidized further with permanganate to phthalic acid. The phthalic acid, on decarboxylation, liberated one carboxyl as carbon dioxide, the isotope content of which corresponded to C_{1,4} in the original phenolic ring. (In view of the symmetry of p-benzoquinone, positions 1 and 4 are indistinguishable by this degradative procedure, the isotope determinations on the carbon dioxide from phthalic acid giving the mean of atoms 1 and 4.) However, since the value of C₁ was known from the previous degradation, it was possible to calculate C_4 in the following way: $C_4 = 2C_{1,4} - C_1$. Also, the isotope content of the 2 equivalent atoms, 2 and 6, follows from C_{2.6} $= 3C_{phenol} - C_{3,5} - C_{1,4}$

EXPERIMENTAL

4-Hydroxybenzoic Acid—475 mg. of tyrosine were added in small portions to a molten mixture of 2 gm. of potassium hydroxide and 2 gm. of sodium hydroxide and kept at 255–265° for 12 minutes, at which time gas evolution had ceased. The reaction vessel was a small silver crucible, heating being carried out with an ordinary Bunsen flame controlled manually. The mixture was cooled and dissolved in a minimum quantity of water, acidified with 50 per cent sulfuric acid and extracted five times with ether. The dried ether extracts were evaporated and the residue was

dissolved in 4 ml. of 1 N sodium hydroxide. A small amount of norit was added; the solution was then filtered and acidified to pH 2 with sulfuric acid. After a short time, 4-hydroxybenzoic acid separated as colorless crystals. The product, after filtration and washing with ice water, was redissolved in alkali, neutralized, and cooled. The crystals, after they had been washed and dried *in vacuo*, melted at 211°, and there was no depression when mixed with authentic 4-hydroxybenzoic acid. Yield; 197 mg.

15 mg. of the acid were used for analysis and combustion to CO_2 . The remaining 182 mg. were dissolved in ether and carefully washed with ether into a solution of 364 mg. of recrystallized 4-hydroxybenzoic acid. The dilution of the preparation, after evaporation of the ether, should be 1:3, which was confirmed by C^{18} and C^{14} determination both before and after dilution. The per cent of C^{13} before and after dilution was 0.183 \pm 0.005 and 0.065 \pm 0.002, respectively, the corresponding C^{14} figures being 129 \pm 5 and 43 \pm 2 c.p.m. per 15 mg. of barium carbonate, which give dilutions of 2.86 \pm 0.10 and 3.09 \pm 0.14. All the following degradations were carried out on the diluted 4-hydroxybenzoic acid, and the isotope contents of the degradation products compared with each other, for which the dilution value of 3.0 was accepted.

3,5-Dinitro-4-hydroxybenzoic Acid – The method of Morgenstern (4) was followed with some modifications. In a small test-tube 50 mg. of the above 4-hydroxybenzoic acid (diluted 1:3) were dissolved at room temperature in 0.27 ml. of concentrated sulfuric acid. The solution was cooled in ice water and an ice-cold mixture of 0.17 ml. of concentrated sulfuric acid and 0.17 ml. of nitric acid, density 1.4, was added. After a short time, the nitration was complete and the reaction mixture was kept at 0° for 2 hours. The crystals were filtered off on a small sintered glass filter, washed with small amounts of ice water, sucked dry, washed with hot benzene, and finally recrystallized from 2 ml. of 70 per cent ethanol. The dry acid melted at 244° and on admixture with an authentic sample melted at 243–244°. Yield, 33 mg.

Picric Acid—110 mg. of 4-hydroxybenzoic acid were converted to 3,5-dinitro-4-hydroxybenzoic acid as before, except that the dinitro acid which crystallized from the acid mixture was not isolated but subjected to further nitration. To the reaction mixture, originally containing 0.90 ml. of concentrated sulfuric acid and 0.35 ml. of nitric acid, density 1.4, an additional amount of 0.35 ml. of nitric acid, density 1.4, was added and the mixture slowly heated on the water bath to 100°. After some foaming (carbon dioxide evolution), the solution became a clear, pale yellow. Heating was continued for 2 hours, when the solution was cooled in ice water, diluted with 3 volumes of ice water, and kept at 0° for 2 hours. The crys-

tals were filtered off, washed with a minimum quantity of ice water, and twice recrystallized from 4 ml. of water. Yield, 56 mg. of picric acid; m.p. 122°; unchanged on mixing with an authentic sample.

Bromopicrin (Tribromonitromethane)—The degradations of 3,5-dinitro-4-hydroxybenzoic acid and picric acid were carried out under identical conditions. In two separate experiments, 32.2 mg. of the dinitro acid and 28.4 mg. of picric acid were dissolved in 1 ml. of water and 10 mg. of calcium hydroxide. 10 gm. of a paste made up from 7.5 gm. of calcium hydroxide, 30 ml. of water at 0°, and 2.5 ml. of bromine were added. After mixing and heating to 40°, the yellow color of the mixture gradually faded and a strong smell of bromopicrin was apparent. The reaction mixture was distilled and 3 ml. of the distillate were collected in a 10 ml. centrifuge tube with a conical tip. The small amount of oily bromopic rin was centrifuged down into the tip of the tube, the supernatant fluid was sucked off, and the oil droplet washed twice by centrifuging with distilled water. Finally, the bromopicrin droplet was transferred by the aid of a micro pipette to a platinum boat and burned in oxygen over a platinum catalyst to carbon dioxide, which was trapped and isolated as barium carbonate. The yield of bromopicrin from 3,5-dinitro-4-hydroxybenzoic acid was 62.7 mg., and from picric acid 67.5 mg. Since the amount of bromopicrin isolated was so small, its purity could not be ascertained, but it melted at about 5°, the recorded melting point being 10°. A control experiment with larger amounts of unlabeled acids gave directly a product with a boiling range of 122-127° (recorded b.p. 127°). The bromine used in the reaction was free from brominated hydrocarbons.

In a separate experiment, 27.8 mg. of picric acid were degraded in a similar way with carbonate-free barium hypobromite, a solution of which was filtered directly into the reaction flask. The bromopicrin was treated as above and the carbon dioxide evolved trapped in barium hydroxide solution. This carbon dioxide represented carbon atoms 2, 4, 6 in the original 4-hydroxybenzoic acid. 82.6 mg. of barium carbonate were obtained, whereas a control experiment run without any nitro compound gave 7.0 mg. Thus 75.6 mg. were obtained from the picric acid and corresponded to a yield of 97 per cent.

p-Benzoquinone—361 mg. of 4-hydroxybenzoic acid were dissolved in 3 ml. of pure, redistilled quinoline; 100 mg. of pure copper powder were added and the mixture was heated for 50 minutes in a metal bath maintained at 255–260°. The 15 ml. reaction flask was connected to a 20 cm. air condenser, the end of which was connected to a centrifuge tube containing filtered, saturated, barium hydroxide solution. Throughout the reaction period, dry, carbon dioxide-free nitrogen was passed through the mixture in order to sweep out the carbon dioxide liberated. The barium

carbonate isolated (480 mg., 94 per cent yield) was analyzed for isotopes and considered to be identical with the β atom of tyrosine.

The phenol remaining in the quinoline solution after completion of the decarboxylation was isolated by adding 10 ml. of 30 per cent sulfuric acid to the reaction mixture, followed by careful extraction with ether. The ether extract was shaken six times with 1 ml. portions of 1 N sodium hydroxide solution and the combined alkaline extracts decolorized with a small amount of norit. After filtration and neutralization with 30 per cent sulfuric acid, a small amount of this solution was treated with bromine water. The tribromophenol was sucked off, dried, and burned to CO₂, trapped as barium carbonate, in order to obtain the isotope value for the sum of the 6 carbon atoms of the ring. The rest of the phenol solution (about 200 mg.) was transferred to a 25 ml. flask, 90 mg. of solid sodium hydroxide and 190 mg. of dry sodium nitrite were added, and the solution was cooled in a freezing mixture to -5° . The nitrosation procedure followed in general the directions given by Bridge (5). 1.68 ml. of a sulfuric acid solution (1 volume of concentrated acid and 5 volumes of water) were added carefully during 5 minutes with cooling and shaking. After addition of the sulfuric acid, the mixture was kept at 0° for 2 hours. during which time most of the 4-nitrosophenol crystallized out as needles.

Without separating the crystals from the mother liquor, the reaction mixture was made alkaline by addition of 3 ml. of ammonia (density 0.91), and hydrogen sulfide was passed in for half an hour. After this time, the solution, which now contained 4-aminophenol and a small amount of sulfur, was made neutral by addition of concentrated sulfuric acid, the liberated hydrogen sulfide being swept out by a stream of nitrogen.

To the solution, now pale yellow and somewhat turbid because of the presence of small amounts of sulfur, 0.3 ml. of concentrated sulfuric acid was added, immediately followed by 50 mg. of vanadium pentoxide and 3 gm. of lead dioxide (PbO₂). The mixture was shaken vigorously for 10 After the first few minutes, the color became dark violet and the temperature rose to about 40°. Later, this color disappeared and, after 10 minutes, the reaction mixture was subjected to steam distillation at 40 mm. pressure, the quinone distilling over into a receiver cooled in a freezing mixture. When the vapors were no longer colored, the condensers were disconnected from the distilling flask and rinsed with small amounts of ether. The total distillate was extracted with ether until the aqueous layer was colorless; the combined extracts were then dried over sodium sulfate and evaporated very carefully to dryness at 40°. The quinone was dissolved immediately in pure pentane at 35° and the solution filtered from a little black, insoluble material and evaporated to 5 ml. After cooling with solid carbon dioxide and acetone, benzoquinone crystallized as

TABLE I

Determined and Calculated Isotope Content of Degradation Products of Tyrosine, and Isotope Content of Individual Atoms of Tyrosine Structure

C_{total} denotes total carbon as barium carbonate by combustion; Ccoon carboxyl carbon. All the measurements were made on duplicate samples. C¹³ is given in atom per cent excess, C¹⁴ in counts per minute per 15 mg. of BaCO₃.

			Isotope values				
Series	Compounds investigated	Position of C atoms in tyrosine	Determi	ined	Calcul	ted	Calculations, based on series
		-	Cn	C14	C19	C14	
A	Tyrosine, Ctotal	All	0.190	153			
		atoms	± 0.005	±6			
			0.201 ± 0.005	٠			
В	" Ссоон	соон	0.090	458			
			±0.005	±8			
C	4-Hydroxybenzoic acid,	Ring, β	0.185	129			
,	Ctotal		±0.005	!			
D	4-Hydroxybenzoic acid, Ссоон	β	0.255	12			
E٠	ССООН	α	±0.005	±5	0.27	17	A, B, C
_		<u> </u>			±0.05	±20	11, 5, 0
\mathbf{F}	Benzene ring	1,2,3,4,	0.190	146			
	$(Tribromophenol, C_{total})$	5,6	± 0.005	±7			
G	Benzene ring	1,2,3,4,			0.18	152	\mathbf{C}, \mathbf{D}
н	Bromopicrin, C _{total} , from	$\begin{bmatrix} 5,6\\1,3,5\end{bmatrix}$	0.191	91	±0.02	±10	
п	picric acid	1,0,0	±0.01	±4			
I	profite Bord	1,3,5			0.17	65	F, L
					± 0.02		,
J	Bromopierin, Ctotal, from	3,5	0.135	122			
	3,5-dinitro-4-hydroxy-		± 0.005	±7			
ĸ	benzoic acid	1			0.27	26	***
v		1			±0.03	± 18	H, I, J
L	CO2 from bromopicrin-	2,4,6	0.21	233	10.00		
	split H	, ,	±0.01	±15			
M	Phthalic acid, Ctotal			88			
	" " Салот			±6			
N	" " Ссоон	1,4	0.140	268			
o		1,4	±0.015	±8		266	F, G, M
١		-, -				±40	-, -, 143
P		4			0.05	506	K, N
ļ					± 0.02	±20	
		4				482	F,G,M,K
		2.6			0.25	±40 60	ECIN
Q	,	2,6			± 0.02	±15	F,G,J,N
R		2,6			0.29	99	L,P
					± 0.03	±40	•

yellow needles, which were filtered off and washed with cold pentane. An additional amount could be obtained from the mother liquor. Total 47.3 mg.; m.p. 113°. The product sublimed without residue.

1,4-Naphthoquinone—The preparation of naphthoquinone was carried out by the method of Fieser (3). 44.5 mg. of p-benzoquinone and 52 µl. of pure, redistilled 1,3-butadiene in 0.27 ml. of glacial acetic acid were kept at room temperature for 44 hours in a thick walled 1 ml. flask fitted with a carefully ground glass stopper. 0.25 ml. of a solution of 4 gm. of sodium dichromate and 0.2 ml. of concentrated sulfuric acid in 2.5 ml. of water were mixed with 0.1 ml. of acetic acid and added to the reaction mixture at 65°. After 30 minutes an additional amount (0.1 ml.) of the

TABLE II

Comparison between Isotope Content of Acetic Acid Used As Single Carbon Substrate for Growth of Torulopsis Yeast and of Individual Atoms of Tyrosine Isolated from Yeast Proteins

 C^{13} and C^{14} values are given in the same units as in Table I. The figures for separate atoms of tyrosine are mean values compiled from the same table.

		C13	C14
Acetic acid of substrate	COOH CH ₃	2.42 ± 0.02	9850 ± 50
Separate carbon atoms of tyrosine	COOH a b 1 2, 6 3, 5 4	$\begin{array}{c} 0.090 \pm 0.005 \\ 0.27 \pm 0.05 \\ 0.25 \pm 0.01 \\ 0.27 \pm 0.03 \\ 0.26 \pm 0.03 \\ 0.135 \pm 0.01 \\ 0.05 \end{array}$	$458 \pm 10 \\ 8 \pm 8 \\ 12 \pm 5 \\ 30 \pm 20 \\ 70 \pm 20 \\ 122 \pm 7 \\ 500 \pm 50$

oxidation mixture was added and the contents of the flask shaken occasionally. At the end of 50 minutes, the flask was cooled and the contents diluted with 3 ml. of ice water and held at 0° for 10 minutes. The pale yellow naphthoquinone was filtered off, washed with water until the washings were colorless, dried *in vacuo* at room temperature, and weighed. Yield, 46.1 mg.; m.p. 121°; mixed m.p. 122°.

Phthalic Acid—5.2 gm. of potassium permanganate and 1.5 ml. of concentrated sulfuric acid were dissolved in 100 ml. of water. To 4.0 ml. of this solution, 43.8 mg. of naphthoquinone were added and the reaction mixture shaken vigorously until the permanganate color had almost disappeared (about 12 minutes). The mixture was extracted continuously for 2 hours with ether. The extract was evaporated to small volume and transferred with careful washing to a 10 ml. centrifuge tube, and the

evaporation was completed in this tube. The crystalline residue was washed twice with 2 ml. of chloroform, twice with pentane, and then dried at 80° for 20 minutes. Yield, 35.5 mg.; m.p. 195–200° with decomposition (recorded m.p. 191–209°); m.p. of anhydride 129° (recorded m.p. 130.5°). The purity of the preparations of phthalic acid from naphthoquinone was checked by model preparations involving larger amounts of substance.

Decarboxylation of phthalic acid was carried out by heating 30.8 mg. of the acid, dissolved in 1.5 ml. of quinoline, with 30 mg. of copper powder for 1 hour at 255°, while a slow current of carbon dioxide-free nitrogen was passed through the reaction flask to sweep out the liberated carbon dioxide. 27.8 mg. of barium carbonate were obtained from the evolved carbon dioxide by trapping the latter in barium hydroxide solution. Determination of the isotope content of the carbonate gave the values for C_{1,4}.

As a control of $C_{1,4}$, a small sample of the phthalic acid (4.3 mg.) was burned to carbon dioxide, which was isolated as barium carbonate. From the isotope content of the latter, $C_{2,4}$ could be calculated from the formula, $C_{1,4} = 8C_{\text{phthalic acid}} - 3C_{\text{phenol}}$.

The results of isotope determinations of the different carbon atoms of tyrosine are shown in Tables I and II.

DISCUSSION

Before considering the question of the biochemical significance of the results obtained from the degradation of tyrosine, a critical examination of the methods is justified. The weakest point, the deduction of C₁, is based on the difference between the two degradations giving rise to bromopicrin, the completeness of which may be questioned. Bromopicrin, obtained from 3,5-dinitro-4-hydroxybenzoic acid, originates from atoms 3 and 5 and, since these 2 carbon atoms are indistinguishable, an incomplete degradation here is unimportant. In the case of picric acid, however, any differences in the proportion of atoms 1, 3, and 5 converted to bromopic in would be reflected in the values for C_{1,3,5} obtained by analysis of a sample of this bromopicrin. On the other hand, previous investigators of this reaction have obtained yields of 95 to 97 per cent bromopicrin from picric acid (6) and this has been confirmed in our own experiments. An alternative method of deducing values for C_{1,3,5} involves the figure obtained for C2.4.6 from the carbon dioxide evolved during the fission of picric acid with barium hypobromite, the following equation being employed: $C_{1,3,5} = 2C_{phenol} - C_{2,4,6}$.

In this way the C^{18} content of $C_{1.8,5}$ was found to be 0.16 atom per cent excess, as compared with the value 0.191 obtained from the bromopicrin produced from picric acid. From these figures, the C^{18} content of C_1 was

calculated to lie between the limits 0.24 and 0.30, the mean value 0.27 \pm 0.03 being accepted. In a similar way, the C¹⁴ content of C₁ could be placed between the limits of 10 and 50 c.p.m. per 15 mg. of barium carbonate, expressed as the mean 30 \pm 20.

If larger amounts of material had been available, reduction of 4-hydroxy-benzoic acid to 4-hydroxybenzyl alcohol, then to p-cresol, might have been tried in an attempt to calculate C₁ directly. p-Cresol, on oxidation by the method of Anker (7), would give acetic acid, the carboxyl of which would be identical with carbon atom 1 of tyrosine and could be isolated by one of several well known degradation procedures.

The uncertainty attached to C₁ is naturally reflected in C₄, although in our case the very high C¹⁴ content of the latter must be regarded as significant. The corresponding C¹³ figures vary between 0 and 0.05, depending on the figures ascribed to C₁. In any case, it could be regarded with safety as "low" and thus the ratio C¹⁴: C¹³ for C₄ is analogous to that for C_{carboxy1}.

The isotope concentration in positions 2 and 6 can be calculated in two ways from the equations

$$C_{2,6} = 3C_{\text{phenol}} - C_{3,5} - C_{1,6}$$

 $C_{2,6} = \frac{1}{2}[3C_{2,4,6} - C_{4}]$

The first is regarded as more accurate, the second being influenced by the errors in C₄. The values of 0.26 ± 0.03 for C¹³ and 70 ± 20 for C¹⁴ might be regarded as fairly reliable.

Table II gives the figures finally accepted for the isotope content of acetic acid used and the several carbon atoms of tyrosine isolated, the errors included being the expected maximum spread and not computed mean errors, which would appear smaller than the figures given.

From the data given in Table II, there appears to be fairly strong evidence that both atom 4 and the carboxyl carbon of tyrosine are derived by more or less direct routes from the carboxyl group of administered acetic acid. The slight excess of C^{13} present in these 2 atoms would arise through recycling of acetate in oxidative breakdown processes, whereby a certain amount of the methyl moiety might be expected to appear in the carboxyl of newly formed acetate. The atoms α , β , 1, 2, and 6, with their rather uniform C^{13} content, seem to be derived from the methyl group of acetate. The "mixed" isotope content of atoms 3 and 5 makes the origin of these 2 atoms uncertain, especially since the C^{13} value of $C_{3,5}$ ought to be higher than 0.135 atom per cent excess if either atom 3 or 5 had come from the methyl group of an acetate molecule, the carboxyl of which would make up atom 4. As a hypothesis, we propose that atom 4 is derived either directly from carbon dioxide or formate or possibly from acetate via

acetoacetate, the carboxyl of the latter making up atom 4 and being followed by ketonic fission. All these possibilities imply that the formation of the ring is connected with the joining up of a carbon atom with two ends of a branched structure which, except for its terminal atom, has been derived mainly from the methyl moiety of acetate.

The sequence of five rather uniformly labeled C^{13} atoms, α , β , 1, 2, and 6, must have been derived from acetate by a series of decarboxylations, perhaps more analogous to the formation of α -ketoglutarate from oxalacetate and acetate than, for instance, head to tail condensation of acetyl residues. Thus, the formation of the benzene ring system of tyrosine does not seem to be a special case of carbon chain formation, as is the production of fatty acids and steroids (cf. Bloch (8)), followed by cyclization, since then an alternating occurrence of C^{13} and C^{14} would have been found in the molecule. It seems possible that the main part of the tyrosine structure may have been derived from an α -keto acid and glycine or an α -keto acid and acetate, followed by amination of a double bond; e.g., as in the formation of aspartate from fumarate.

Finally, we would like to point out that these results and their interpretation may apply only to *Torulopsis* yeast or, at least, to the lower fungi. The experiments would have to be repeated on a variety of biological materials before any general conclusions could be drawn.

Work is in progress at present on a similar degradation of phenylalanine with the object of correlating the metabolism of phenylalanine with tyrosine.

SUMMARY

A method has been developed which enables the following carbon atoms of tyrosine to be isolated in the form of carbon dioxide: the carboxyl, β , 3 and 5, 1 and 3 and 5, 2 and 4 and 6, and, finally, 1 and 4. Tyrosine obtained from a protein hydrolysate from *Torulopsis* yeast, grown on labeled acetic acid as a single carbon source, was degraded by this method and the isotope content of each carbon atom of the tyrosine determined.

The carboxyl group of administered acetic acid appears as carboxyl and the ring atom 4 of tyrosine, while the methyl group contributes mainly to α , β , 1, 2, and 6 atoms. The origin of atoms 3 and 5 is uncertain.

The mechanism of tyrosine synthesis is discussed.

Our thanks are due to the Swedish State Scientific and Medical Councils for financial support of this work.

BIBLIOGRAPHY

- Baddiley, J., Ehrensvärd, G., Johansson, R., Reio, L., Saluste, E., and Stjernholm, R., J. Biol. Chem., 183, 771 (1950).
- Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., J. Biol. Chem., 150, 251 (1943).
- 3. Fieser, L. F., J. Am. Chem. Soc., 70, 3165 (1948).
- 4. Morgenstern, O., Monatsh. Chem., 31, 285 (1910).
- 5. Bridge, J. L., Ann. Chem., 277, 79 (1893).
- 6. Will, W., Ber. chem. Ges., 47, 962 (1914).
- 7. Anker, H. S., J. Biol. Chem., 166, 219 (1946).
- 8. Bloch, K., Physiol. Rev., 27, 596 (1945).

COMBINATION OF WOOL PROTEIN WITH CATIONS AND HYDROXYL IONS*

By JACINTO STEINHARDT AND ETHEL M ZAISER

(From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge)

(Received for publication, September 20, 1949)

Earlier work with two proteins, wool and egg albumin, and over forty different strong acids (13, 15, 19, 20) has shown that their titration curves differ widely (up to 2.5 units) with respect to the pH axis. This effect was shown to arise from combination of proteins with anions as well as with hydrogen ions; the differences between the positions of the respective titration curves permit calculation of the relative affinities of the anions for the protein (14, 19). The relative affinities may also be deduced from the effectiveness of the anions in catalyzing hydrolysis of the amide and peptide bonds of the protein (16). Anions of moderate or high affinity, when combined on the primary or secondary amides, catalyze their hydrolysis; the catalytic effect is approximately proportional to their affinity for the protein as calculated from the titration curve.

The concept of anion binding by proteins has now been amply confirmed by work in many other laboratories (3, 4, 6, 8, 9, 23–25) by dialysis equilibrium and other methods (9) which do not depend on the interpretation of titration phenomena. The effect of added salt on the titration curve of wool is, in part, another manifestation of the combination with anions (17). Since the effect of salt on the acid titration is exactly paralleled by its effect on titration with bases, this particular protein must bind cations also, although evidence has been presented which suggests that two other proteins, serum albumin (9) and aldolase (24), may bind anions only.

If wool binds cations, the titration curves obtained with different strong bases should also differ from one another. The present investigation determines the extent to which this inference is sustained, and explores the relation of the affinity of cations for this protein to the size or shape of the ions tested.

Accurate measurements with bases are more difficult to make than with acids. This is especially true when one must use very dilute solutions (down to 10^{-5} M) and in the absence of the buffering capacity of dissolved protein. This results in extreme susceptibility to contamination by at-

* Brief accounts of these measurements were reported at the meetings of the American Society of Biological Chemists on April 19, 1949, at Detroit, and at a meeting of the Washington Chemical Society on May 14 at College Park, Maryland.

mospheric CO₂, and to effects due to dissolving of or adsorption by glass. The following factors also limit the accuracy and possible range of the measurements with base, and are dealt with later. (a) Measurements of pH values with the glass electrode in the alkaline range are subject to several sources of error which must be minimized or calibrated. (b) Wool, like other sulfur-containing proteins, is sensitive to even very dilute alkali unless exposed for short intervals (consistent with attainment of diffusion equilibrium), at the lowest temperatures possible. (c) The choice of univalent bases is limited, compared with the wide choice of strong acids previously investigated. Few inorganic ions are available (Li⁺, K⁺, Cs⁺, and Rb⁺, since Na⁺ must be omitted because of its effect on the glass electrode). Besides these, there are only the quaternary ammonium compounds. No attempt has been made, therefore, to calculate affinity constants, or to study the stoichiometry of the reactions.

EXPERIMENTAL

Except as specifically noted, the procedures employed in the purification of the wool, determination of the alkali equivalence of its ash, and measurement of the base bound were as previously described (17, 18).

Wool¹—The ash content was 0.25 per cent. A correction of 0.042 m.eq. per dry gm., the alkali equivalence of the cationic ash as determined by electrodialysis (11), was added to the measured amount of base bound. Corrected measurements of the combination with hydrochloric acid agreed within the experimental error with earlier results (17). All results refer to dry weight of wool.

Bases—The bases used were commercial products of c.p. grade, except as described below, and were free of carbonate. Tetraalkylammonium bromides or iodides were converted into hydroxides by treating their solutions with excess silver oxide in the cold. Quaternary ammonium salts which were not available commercially were synthesized from alkyl bromides and trimethylamine by the method of Scott and Tartar (10). The molecular weights of the salts, used as a measure of their purity, differed from the theoretical values by less than a few tenths per cent, except for the octyltrimethylammonium bromide, which was 2 per cent high.

Measurement of Base Combined—The method of measurement has been described earlier (17). After the initial equilibration period, the alkaline solution withdrawn for analysis was replaced in some cases with distilled water. During a second period for equilibration the wool invariably gave up base to the solution, and a second titration curve was obtained whose coincidence with the first was proof that equilibrium had been attained.

¹ Thanks are due to Dr. Milton Harris of the Harris Research Laboratories, Washington, D. C., for providing this material.

When the two curves did not coincide, as with tetradecyltrimethylammonium hydroxide, they set narrow limits to the range within which the true equilibrium curve must fall.

Measurements made at high base concentrations were corrected for the decomposition of disulfide bonds (18). The correction for the amount of protein dissolved was less than 2 per cent of the base bound in the worst cases (long exposure to concentrated base) and was usually negligible. The extent of decomposition of the wool depended on the concentration of base and the time of exposure, and was independent of the identity of the base, with one well marked exception: tetrabutylammonium hydroxide caused less than half as much decomposition as any other base. There was no evidence of catalysis of hydrolysis by cations, but the highest amounts bound represented a smaller fraction of the maximum than was required for catalysis by anions (16).

Measurement of pH-In the present investigation it was necessary to make pH measurements corresponding to base concentrations as high, on occasion, as 0.06 m. Since the glass electrode in alkaline solutions is subject to substantial errors, and these errors depend, in part, on the base employed, an empirical calibration procedure was resorted to for each base This procedure proved satisfactory, because the deviations of the behavior of a glass electrode from that of an ideal hydrogen electrode are very much smaller and more reproducible at 0°, in the absence of salts, than at room temperature when even small amounts of alkali salts are The maximum difference between indicated pH (calculated from the c.m.f. as if a hydrogen electrode were used) and true pH never exceeded about 0.2 unit, and this maximum correction was applied only at values well above pH 13. All the corrections determined could be taken into account by reading off pH values from a linear plot of e.m.f. increments against pH values calculated from the expression $-\log f_{\text{BOH}}C_{\text{BOH}}$ corresponding to a series of dilutions of each base (Fig. 1). This procedure, although resting on a somewhat more secure foundation than in earlier work. is essentially similar to the one previously used (18).

The calculation of pH values required for the calibration depends on values of f_{BOH} which have been taken from the literature (2), or calculated from osmotic coefficients (5). In the absence of earlier data, f_{BOH} was calculated by means of the Debye-Hückel limiting law, since, in dilute solutions, hydroxylic compounds of large cations will probably show even smaller deviations from ideality than are given by this relation.² Resulting uncertainties in pH will not exceed 0.03 unit in the most concentrated range, and should be negligible below pH 13.

² Personal communication from Professor G. Scatchard.

No standards corresponding to concentrations of base much below 10⁻³ m were used. Such dilute unbuffered solutions of bases are too susceptible to contamination (by CO₂, dissolved glass, etc.) to be considered pH standards at all. The validity of the pH values of the most dilute solutions, read from the straight line established by borate buffer and by points between pH 12

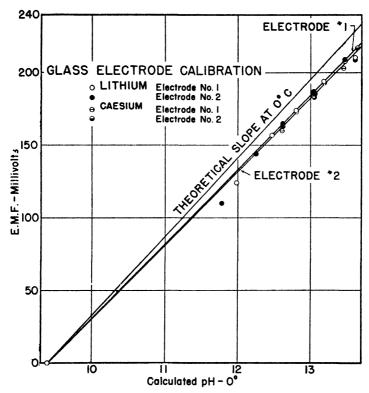


Fig. 1. Glass electrode calibrations for lithium hydroxide and cesium hydroxide. The e.m.f. increments were measured from a standard 0.05 m borate buffer (pH 9.39 at 0°) with two different glass electrodes. The calibration curves for the two bases are indistinguishable.

and 13.6, is confirmed by indicator readings, and by the observation (7) that the difference in potential of a glass electrode and hydrogen electrode is constant in unbuffered solutions between pH 6.0 and 8.5.

RESULTS AND DISCUSSION

Since differences in the position of the base titration curves with respect to the pH axis have been found of comparable magnitude to those previously demonstrated on the acid side, it appears that large differences

in the affinity of cations for wool protein parallel the affinity differences which exist for anions.

The results of a representative pair of experiments illustrating the shift in position of the curve are shown in Fig. 2. This figure also shows a number of features common to all the other experiments, as indicated in the legend. The corrections applied to the KOH data for disulfide decomposi-

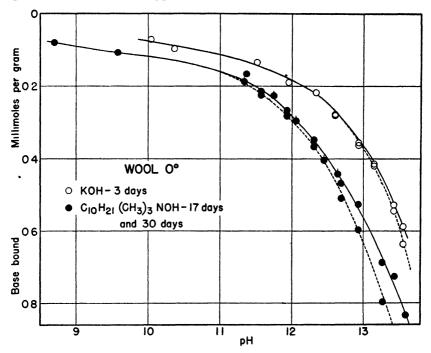


Fig. 2. Combination of wool protein with a representative pair of strong bases at 0°. The dotted lines represent uncorrected data, and the solid lines data corrected for the decomposition of disulfide bonds. The tagged circles represent measurements obtained upon reversal of the combination of protein with base. Note the large non-uniform shift in the curve for decyltrimethylammonium hydroxide.

tion are very small, since the protein was exposed to the solutions of base for only 3 days. At pH values below 13, the corrections may be neglected. With decyltrimethylammonium hydroxide, the wool was exposed for 17 days, and much larger amounts of decomposition resulted, noticeable at pH values as low as 12; however, the experimentally determined corrections result, when applied, in a smooth set of data. Some of the points (those which are tagged) represent measurements made after an additional 13 days immersion in solutions diluted with water after the initial period. These points, corrected for additional protein decomposition, are wholly con-

sistent with the 17 day measurements. They demonstrate, therefore, that (a) the correction for decomposition is made correctly and (b) that the solid line represents the attainment of a truly reversible acid-base equilibrium.

The results obtained with six homologous compounds, alkyltrimethylammonium hydroxides, are shown together in Fig. 3. As the molecular

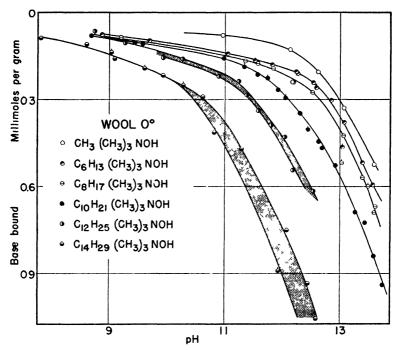


Fig. 3. Combination of wool protein with six homologous alkyltrimethylammonium hydroxides at 0°. The shaded areas represent the range of uncertainty in the equilibrium position of the curve, due to failure to attain final equilibrium in the long times (15 to 17 days) allowed for the two highest homologues. The tagged circles represent measurements obtained upon reversal of the combination of protein with base.

weight (and the length of the alkyl chain) increases, there is a progressive displacement of the titration curves to lower pH values; i.e., more base is bound at lower base concentrations. The shift corresponding to each 2-carbon increment in chain length also becomes progressively larger. Thus, there is only a small difference between the results obtained with the lower homologues, but there is a very substantial difference between the decyl and dodecyl derivatives and an even larger difference between the dodecyl and tetradecyl. Similar shifts in acid titration curves have

been shown to be the result of differences in the affinity of anions for the protein, and have been used to calculate these differences (14, 19). No such calculation is made here, because the complex nature of the titration curve in the alkaline region³ would make the calculation somewhat arbitrary, but the qualitative conclusions are plain.

A striking feature of the data obtained with the decyl and tetradecyl derivatives is the large amount of base bound, 0.94 mm per gm. at pH 13.8 for the former compound and over 1.0 mm at pH 12.0 for the latter. Both · figures exceed 0.8 mm per gm., accepted by earlier authors (12), even though the present curves give no indication of leveling off to a maximum value. On the basis of analytical data, the theoretical maximum base-binding capacity of wool is 1.18 mm per gm., if the content of tyrosine, 0.32 mm per gm., is added to the sum of the arginine and lysine, 0.86 mm per gm. (the latter is very nearly equal to the dicarboxylic acid content indicated by the acid titration curve). The assumption that the cationic form (RNH₃⁺) of arginine is too weak an acid to be titrated in this range would reduce the maximum base bound to 0.59 mm per gm. This figure approximates the value found with the more common bases, in solutions containing about 0.05 to 0.07 molal free base. Thus, it appears that the electrostatic effect of combination of the protein with cations has weakened the proton affinity of arginine sufficiently to make it dissociate with low concentrations of base.

Similar effects must be produced on the cationic form of lysine, and on undissociated tyrosine. It will be shown below that the effect on lysine is much greater than the effect on tyrosine or arginine.

One cannot distinguish, within a single homologous series of cations, between differences in affinity due to their total mass, linear dimensions, or other spatial relationships, or to more specific chemical effects. In Fig. 4, some of the results already shown are compared with data for more symmetrical quaternary ions with molecular weight in the same range. The data obtained with tetraethylammonium hydroxide, which has only a slightly lower molecular mass than hexyltrimethylammonium hydroxide, show a significantly lower affinity for the symmetrical cations. Much more marked differences appear when tetrabutylammonium hydroxide is compared with an unsymmetrical molecule of approximately the same molecular weight, dodecyltrimethylammonium hydroxide. Here, there is a displacement to lower pH values of well over 1 pH unit (higher affinity)

³ The curve of combination with acid can be characterized by a single pair of equilibrium constants, one for the dissociation of a single type of acid group (COOH), and one for the corresponding association with anions (14, 19). The base curve results from the dissociation of at least three proton donors (in lysine, arginine, and tyrosine) (1).

for the asymmetric cation, although the curves tend to come together for small amounts of base bound.

Data for a less asymmetric molecule, phenyltrimethylammonium hydroxide, are also shown in Fig. 4. The molecular weight of this compound is comparable with that of the more asymmetric hexyltrimethylammonium hydroxide, and the more symmetrical tetraethylammonium hydroxide. The results obtained are practically identical with the asymmetric ion at large amounts bound (shift in pH), but unlike the completely symmetric

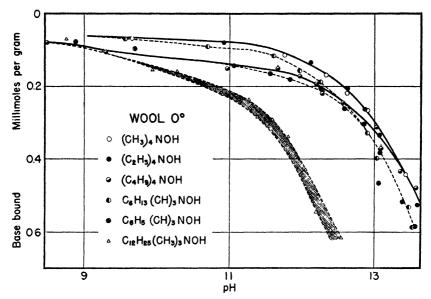


Fig. 4. Combination of wool protein with symmetric and asymmetric quaternary ammonium hydroxides at 0°. The tagged circles represent measurements obtained upon reversal of the combination of protein with base.

ions, the shift in pH is still great for small amounts bound. The reason for this marked specificity remains obscure.

Experiments with univalent inorganic bases show only small differences (Fig. 5), although the spread in molecular weight is relatively large. All of the inorganic cations tested, however, had slightly higher affinities for wool than the organic cation of lowest molecular weight, tetramethylammonium.

Close examinations of the preceding figures will show that the shift in pH produced by the cations of higher affinity is not uniform along the entire curve, as it was in the case of titration with acid (19, 20); i.e., the base curves are not exactly superimposable. The differences in displacement are made clear in Fig. 6 in which the displacements in pH for all bases

(relative to the curve for tetramethylammonium hydroxide) are compared at various amounts bound. A large number of bases (predominantly those of low molecular weight) produce a relatively small displacement (<0.4 unit) over most of the titration curve, but all except the smallest of these produce a very much larger displacement when only small amounts (<0.2 mm per gm.) are bound. This larger shift occurs in the region of the titration curve corresponding to the dissociation of lysine. As the ion size increases, especially among the detergent (asymmetric) ions, much larger displacements are found all along the titration curve, but the lysine region continues to be displaced to a larger extent, about 1 pH unit more

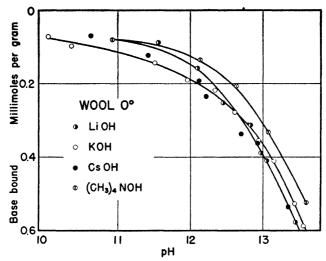


Fig. 5. Combination of wool protein with inorganic bases at 0°. The curve for the quaternary ammonium base of lowest molecular weight is inserted for comparison.

than the rest of the titration curve. The symmetrical tetrabutylammonium ion, however, behaves uniquely in this respect; it shows a maximum displacement of almost 2.5 pH units at about 0.1 mm bound, but no displacement at all at larger amounts (>0.4 mm per gm.) bound. Its selective effect on the lysine component is extremely sharp; in fact the shift in the lysine component which it produces is as great as the shift produced by the asymmetric ions of similar molecular weight, although its effect on the other components is negligible.⁴

⁴ The fact that ions of relatively low detergency, phenyltrimethylammonium and tetrabutylammonium, are outstanding in the selectivity of their affinity effects in the lysine region tends to support the view that this effect is real, and not an artifact brought about by surface adsorption effects at the electrode.

The inclusion in Fig. 6 of data obtained with KOH in the presence of 1 m formaldehyde demonstrates the close parallel between the selective effect of large cations on the dissociation of lysine groups and the wholly

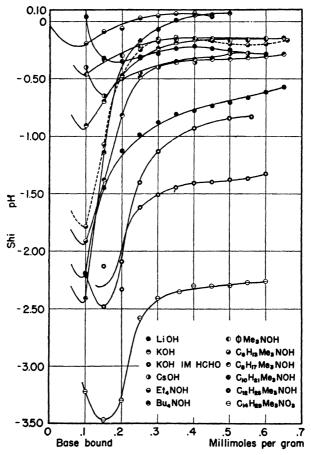


Fig. 6. Variation of shift in pH with amount of base bound for twelve bases. A curve showing the similar effect of formaldehyde is included for comparison. Displacements in pH are referred to the titration curve of tetramethylammonium hydroxide, which has the lowest affinity of any of the bases tested.

similar effect of formaldehyde (22) which is known to be predominantly on lysine.

The selective effect of cations on the lysine component just described had no parallel in the earlier data obtained with acids. Although other causes for this effect are conceivable, it may merely indicate that the first cations which combine are bound more strongly than those which combine only

when the base concentration is increased. If this is the case, the effect is not on lysine at all, but manifests itself on the lysine component of the titration curve only because the first considerable numbers of protons to dissociate, on addition of base, come from lysine ammonium groups, the strongest acidic groups remaining in the molecule. This explanation is consistent with other work (4, 9) which indicates that, with other proteins, a certain number of ions combine much more readily than the remainder. It is a necessary consequence of this explanation, however, that, with wool, all the anions which can combine are bound essentially equally tightly.

Forces Involved in Ion Binding by Protein

The results with anions reported earlier (13, 15, 19, 20) established that, although non-specific (i.e. van der Waals) forces, rather than primary chemical valences, were primarily responsible for the binding of anions by wool and egg albumin, the effect of these forces manifested itself only when long range (i.e. coulombic) forces increased the probability that the ions would approach closely enough for the van der Waals forces to operate (see (21) p. 422, foot-note). The principal evidence is summarized below, where it is compared with evidence from the present investigation.

Van der Waals Forces Involved—The affinity of anions has been shown to depend predominantly on molecular weight. The same relationship has been established in this paper for cations; and it has further been shown that molecular asymmetry can greatly enhance the affinity at a given molecular weight. Since this fact suggests that the size of the ion along its longest axis may be the determining factor, the data obtained with cations have been plotted against both molecular weight and length of major axis, as measured on fully extended Fischer-Hershfelder models. These comparisons are shown in the top and bottom portions respectively of Fig. 7. All of the data obtained with organic cations can be expressed as a smooth monotonic function of ion size, although they scatter widely (see especially tetrabutylammonium ion) when plotted against molecular The inorganic ions, however, have higher affinities per unit weight or size, than do the organic ions; these ions, undoubtedly, present quite different surfaces to the protein; they alone are not covered with molecularly linked hydrogen.

Organic ions of small size (molecular weights below about 150 or sizes below about 12 to 14 A), differ very little in affinity among one another. Above these limits, the affinity increases rapidly, causing a displacement in pH, for a given amount bound, of about 1 unit for every increment in molecular weight of 45 (for asymmetric ions). Among the anions studied previously, the value at which a strong dependence on molecular weight begins is the same or slightly smaller, and the affinity increases about half

as much per unit of molecular weight. The existence of minimum sizes for differences in affinity in both cases is similar to the conditions for micelle formation in compounds containing these ions.

Long Range Coulombic Forces Also Involved—This may be inferred from the fact that the combination with anions was stoichiometric and the max-

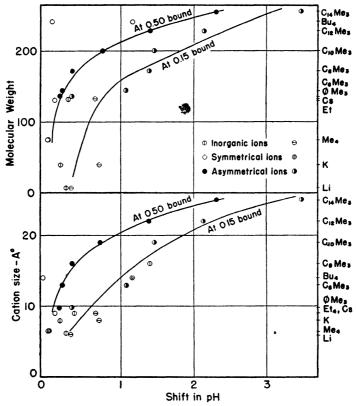


Fig. 7. Relation of shift in pH to molecular weight and length of cations. Curves are given for two different amounts of base bound to allow for the differences in effects produced at small and large amounts bound.

imum amount combined corresponded to the number of positively charged groups. When an apparent excess combination occurred (i.e. more than would be accounted for by back titration of COO⁻ groups), it could be shown that hydrogen ion also combined stoichiometrically with amide groups (RCONH₃+) of glutamine and asparagine, preserving electrical neutrality. The fact that this slight excess combination, was actually with the amide groups was shown by the experimental demonstration that hydrolysis of these groups always set in at the lowest concentrations of

acid (different with each anion) at which the excess combination occurred (16).

In the experiments with base, stoichiometry cannot be demonstrated, since the maximum theoretical amounts of base were never bound within the range of concentrations employed. However, the fact that these maxima were only approached, and never exceeded, makes it unnecessary to assume that the cations combined, by van der Waals forces alone at uncharged sites, or by building up micellar layers on the first cations to combine at charged sites. Thus, it appears that, as with anions, the van der Waals forces alone are not sufficient to overcome the thermal energy dissociating these combinations, except in the vicinity of long range electrostatic forces which reinforce their effect.

Specific Chemical Bonds Not Involved—The best evidence for this thesis is the demonstration, in the work with anions, that the binding energy depended primarily on molecular size, with only secondary differences between non-homologous compounds. A much smaller range of chemical structures has been available with cations, but the results have been much the same. Furthermore, if specific chemical forces (sharing of electrons) are responsible at all, a complete electron pair (donated, for example, to the protein by the anion) would have to be involved. The formation of such a dative bond in the vicinity of the RNH₃+ groups of the protein is impossible because all the atoms of these groups already have complete valence shells. Although the anions might donate an electron pair elsewhere in the protein secule, the organic cations used in this study could neither donate nor accept pairs. Since cations and anions of the same size have very similar affinities for the protein, the formation of covalent bonds (other than hydrogen bonds) cannot be involved.

Thanks are due to Professor George Scatchard of the Department of Chemistry, Massachusetts Institute of Technology, for extension of the Department's facilities, and for many helpful discussions.

SUMMARY

The titration curves of wool at 0° with twelve strong monovalent bases exhibit similar wide differences with respect to position on the pH axis, as has been previously demonstrated with acids. Thus, as with anions, the affinity of cations for protein increases with increasing molecular dimensions. Among organic cations of the same weight, asymmetric ions produce much the larger shift. Unlike the effects of anions, which are uniform over the entire acid curve, the effect of cations is more marked on the portion of the titration curve which is also affected by formaldehyde; i.e., the part due to lysine. The maximum shift in position of this part of the titration curve, obtained with tetradecyltrimethylammonium hydrox-

ide, exceeds 2 pH units. Marked effects on other portions of the curve are shown only by asymmetric ions larger than the hexyltrimethylammonium ion. Explanations of this distinction have been suggested, as well as of the selective effect on the dissociation of lysine. As with anions, the binding of cations is brought about by van der Waals forces which have an opportunity to manifest themselves only when long range (electrostatic) forces increase the probability of close enough approach. No specific chemical bonds are involved.

BIBLIOGRAPHY

- Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York (1942).
- Harned, H. S., and Owen, B. B., The physical chemistry of electrolytic solutions, New York, 369 (1943).
- 3. Karush, F., and Sonenberg, M., J. Am. Chem. Soc., 71, 1369 (1949).
- Klotz, I. M., Walker, F. M., and Pivan, R. B., J. Am. Chem. Soc., 68, 1486 (1946).
 Klotz, I. M., and Walker, F. M., J. Am. Chem. Soc., 69, 1609 (1947).
 Klotz, I. M., Triwush, H., and Walker, F. M., J. Am. Chem. Soc., 70, 2935 (1948).
 Klotz, I. M., and Urquhart, J. M., J. Am. Chem. Soc., 71, 847, 1597 (1949).
- 5. Lange, J., Z. phys. Chem., Abt. A, 168, 147 (1934).
- 6. Luck, J. M., and Schmidt, A. S., Stanford Med. Bull., 6, 133 (1948).
- 7. MacInnes, D. A., and Belcher, D., J. Am. Chem. Soc., 53, 3315 (1931).
- Putnam, F. W., and Neurath, H., J. Am. Chem. Soc., 66, 1992 (1944); J. Biol. Chem., 159, 195 (1945).
- Scatchard, G., and Black, E. S., J. Phys. and Colloid Chem., 53, 88 (1949); Ann. New York Acad. Sc., 51, 660 (1949).
- 10. Scott, A. B., and Tartar, H. V., J. Am. Chem. Soc., 65, 692 (1942).
- Sookne, A. M., Fugitt, C. H., and Steinhardt, J., J. Res. Nat. Bur. Standards, 25, 61 (1940).
- 12. Speakman, J. B., and Hirst, M. C., Tr. Faraday Soc., 29, 148 (1933).
- 13. Steinhardt, J., Ann. New York Acad. Sc., 41, 287 (1941).
- 14. Steinhardt, J., J. Res. Nat. Bur. Standards, 28, 191 (1942).
- 15. Steinhardt, J., Ann. Rev. Biochem., 14, 145 (1945).
- 16. Steinhardt, J., and Fugitt, C. H., J. Res. Nat. Bur. Standards, 29, 315 (1942).
- Steinhardt, J., Fugitt, C. H., and Harris, M., J. Res. Nat. Bur. Standards, 24, 335 (1940).
- Steinhardt, J., Fugitt, C. H., and Harris, M., J. Res. Nat. Bur. Standards, 25, 519 (1940).
- Steinhardt, J., Fugitt, C. H., and Harris, M., J. Res. Nat. Bur. Standards, 26, 293 (1941).
- Steinhardt, J., Fugitt, C. H., and Harris, M., J. Res. Nat. Bur. Standards, 28, 201 (1942).
- Steinhardt, J., Fugitt, C. H., and Harris, M., J. Res. Nat. Bur. Standards, 29, 417 (1942).
- 22. Steinhardt, J., Fugitt, C. H., and Harris, M., J. Biol. Chem., 165, 285 (1946).
- 23. Teresi, J. D., and Luck, J. M., J. Biol. Chem., 174, 653 (1948).
- 24. Velick, S. F., J. Phys. and Colloid Chem., 53, 135 (1949).
- 25. Volkin, E., J. Biol. Chem., 175, 675 (1948).

INDEX TO AUTHORS

	Bendich, Aaron, Brown, George
Allen Frank Westhington Co. Buckey	Bosworth, Philips, Frederick S., and
Allen, Frank Worthington. See Bacher and Allen, 633, 641	Thiersch, J. B. The direct oxidation
Allison, James B. See Roth and Allison,	of adenine in vivo, 267
173	See Geren, Bendich, Bodansky, and
Anker, H. S. See Pihl, Bloch, and	Brown, 21
Anker, 441	Berg, Clarence P. See Van Pilsum and
Archibald, Reginald M. See Phillips,	Berg, 279
Van Slyke, Hamilton, Dole, Emerson,	Bloch, Konrad. See Little and Bloch,
and Archibald, 305	See Pihl and Bloch, 431
See Van Slyke, Hiller, Phillips,	See Pihl, Bloch, and Anker, 441
Hamilton, Dole, Archibald, and Eder,	Bodansky, Oscar. See Geren, Bendich,
331	Bodansky, and Brown, 21
See Van Slyke, Phillips, Dole,	Boissonnas, R. A. See Keller, Bois-
Hamilton, Archibald, and Plazin, 349	sonnas, and du Vigneaud, 627
Awapara, Jorge, Landua, Alton J.,	Bordeaux, Julien. See Lorber, Lifson,
and Fuerst, Robert. Free amino-	Sakami, and Wood, 531
ethylphosphoric ester in rat organs	See Lorber, Lifson, Wood, Sakami,
and human tumors, 545	and Shreeve, 517
Axelrod, A. E. See Hofmann, Dickel,	Boström, Harry. See Jorpes, Boström, and Mutt, 607
and Axelrod, 481	Boyer, Paul D. See Rabinovitz and
	Boyer, 111
В	Bridwell, E. G. See Harger, Raney,
Dacher Tames E and Allen French	Bridwell, and Kitchel, 197
Bacher, James E., and Allen, Frank Worthington. A comparison of pen-	Brinkman, John H. See Shantz and
tose nucleic acid from pancreas with	Brinkman, 467
ribonucleic acid from yeast, 641	Brown, Douglas M. See Smith and
— and —. The composition of certain	Brown, 241
fractions obtained by the action of	Brown, George Bosworth. See Bendich, Brown, Philips, and Thiersch, 267
ribonuclease on ribonucleic acid from	See Furst, Roll, and Brown, 251
yeast, 633	See Geren, Bendich, Bodansky, and
Baddiley, J., Ehrensvärd, G., Johansson,	Brown, 21
R., Reio, L., Saluste, E., and	Bush, Jess A. See Stevens and Bush,
Stjernholm, R. Acetic acid metab-	139
olism in Torulopsis utilis. I. The cultivation of Torulopsis yeast on	C
C ¹³ H ₂ C ¹⁴ OOH as the single carbon	Cagan, Ralph N., Gray, John L., and
source, 771	Jensen, H. The influence of certain
-, -, Klein, E., Reio, L., and Saluste,	endocrine secretions on amino acid
E. Acetic acid metabolism in Toru-	oxidase, 11
lopsis utilis. II. Metabolic connec-	Camien, Merrill N., and Dunn, Max S.
tion between acetic acid and tyrosine	An improved microbiological method
and a method of degradation of the	for the determination of cystine in

teroides P-60,

777

human urine with Leuconostoc mesen-

561

phenolic ring structure in tyrosine,

804 INDEX

Canzanelli, Attilio, Rapport, David, and Guild, Ruth. The effect of previous carbohydrate deprivation on the synthesis of alanine by liver in vitro, Chambers, Dorris C. See Spies and Chambers, Chandler, Joseph P. See Mackenzie, Rachele, Cross, Chandler, and du Vigneaud, — See Simmonds, Keller, Chandler, and du Vigneaud, 191 Cohen, Philip P. See McGilvery and Cohen, Cook, Margaret. See Lorber, Lifson, Sakami, and Wood, Sakami, and Wood, Sakami, and Shreeve, 517	Dunn, Max S. See Camien and Dunn, 561 E Eder, Howard A. See Van Slyke, Hiller, Phillips, Hamilton, Dole, Archibald, and Eder, 331 Ehrensvärd, G. See Baddiley, Ehren- svärd, Johansson, Reio, Saluste, and Stjernholm, 771 —. See Baddiley, Ehrensvärd, Klein, Reio, and Saluste, 777 Eichel, Bertram, Wainio, W. W., Per- son, P., and Cooperstein, S. J. A par- tial separation and characterization of cytochrome oxidase and cytochrome b, 89 Elion, Gertrude B. See Hitchings,
Cooper, Betty J. See McConnell and	Elion, Falco, Russell, Sherwood, and
Cooper, 459 Cooperstein, S. J. See Eichel, Wainio, Person, and Cooperstein, 89 Copenhaver, J. H., Jr., McShan, W. H., and Meyer, Roland K. The determination of glutamic acid dehydrogenase in tissue homogenates, 73 Cross, Nancy. See Mackenzie, Rachele, Cross, Chandler, and du Vigneaud, 617	VanderWerff, Elvehjem, C. A. See Potter and Elvehjem, See Williams and Elvehjem, See Williams and Elvehjem, Van Slyke, Hamilton, Dole, Emerson, and Archibald, Engel, Lewis L., Patterson, Helen R., Wilson, Hildegard, and Schinkel, Marion. The colorimetric estimation of urinary neutral steroid alcohols, 47
Dickel, Daniel F. See Hofmann, Dickel,	F
and Axelrod, 481 Dill, Wesley A. See Glazko, Dill, and Rebstock, 679	Falco, Elvira A. See Hitchings, Elion, Falco, Russell, Sherwood, and Vander- Werff,
Dische, Zacharias. A modification of the carbazole reaction of hexuronic acids for the study of polyuronides, 489	Feldstein, Milton. See Klendshoj, Feldstein, and Sprague, 297 Findlay, Stephen P., and Dougherty, Gregg. The activity of certain sub-
Dole, Vincent P. See Phillips, Van Slyke, Hamilton, Dole, Emerson, and	stituted indoleacetic acids as plant hormones in the pea test. 361

305

331

349

and

361

Dole,

Archibald.

Dougherty,

See Van Slyke, Hiller, Phillips,

Hamilton, Dole, Archibald, and Eder,

See Van Slyke, Phillips,

Hamilton, Archibald, and Plazin,

Dougherty, Gregg. See Findlay

See Baddiley, Ehrenson, Reio, Saluste, and ey, Ehrensvärd, Klein, uste, 777 , Wainio, W. W., Perooperstein, S. J. A parand characterization of idase and cytochrome b, Hitchings, B. See Russell, Sherwood, and 1 A. See Potter and 587 ns and Elvehjem, 539 lall, Jr. See Phillips, amilton, Dole, Emerson, , Patterson, Helen R., egard, and Schinkel. colorimetric estimation tral steroid alcohols, 47 F See Hitchings, Elion, , Sherwood, and Vandern. See Klendshoj, Feldprague, en P., and Dougherty. activity of certain subleacetic acids as plant the pea test. 361 Foster, John C. See Woodruff and Foster, 569 Fuerst, Robert. See Awapara, Landua, and Fuerst, Furst, Sidney S., Roll, Paul M., and Brown, George Bosworth. On the renewal of the purines of the desoxypentose and pentose nucleic acids. 251

805 AUTHORS

William. Bendich, Aaron. Bodansky, Oscar, and Brown, George Bosworth. The fate of uric acid in Glazko, Anthony J., Dill, Wesley A., and Rebstock. Mildred C. Biochemstudies on chloramphenicol (Chloromycetin). III. Isolation and identification of metabolic products 679 in urine. Granick, S. Magnesium vinyl pheoporphyin as, another intermediate in the biological synthesis of chlorophyll, 713 Gray, John L. See Cagan, Gray, and 11 Jensen, Green, Harry. See Meyerhof and Green, 377 Greenman, Lawrence. Alterations in blood glucose following intravenous 577 galactose. Guild. Ruth. See Canzanelli, Rapport, and Guild, 291 H Hamilton, Paul B. See Phillips, Van Archibald, Hamilton, Dole, Archibald, and Eder, 331

Slyke, Hamilton, Dole, Emerson, and 305 See Van Slyke, Hiller, Phillips,

- See Van Slyke, Phillips, Dole, Hamilton, Archibald, and Plazin,

349

Hammarsten, Einar, Reichard, Peter, and Saluste, E. Pyrimidine nucleosides as precursors of pyrimidines in polynucleotides,

Harger, R. N., Raney, B. B., Bridwell, E. G., and Kitchel, Mary F. The partition ratio of alcohol between air and water, urine and blood; estimation and identification of alcohol in these liquids from analysis of air equilibrated with them,

Helmreich, M. L. See Samuels, Sweat, Levedahl, Pottner, and Helmreich,

Heppel, Leon A., and Hilmoe, R. J. Metabolism of inorganic nitrite and

nitrate esters. II. The enzymatic reduction of nitroglycerin and erythritol tetranitrate by glutathione, 129 Hiller, Alma. See Van Slyke, Hiller, Phillips, Hamilton, Dole, Archibald, and Eder,

Hilmoe, R. J. See Heppel and Hilmoe, Hitchings, George H., Elion, Gertrude

B., Falco, Elvira A., Russell, Peter B., Sherwood, Marion B., and Vander-Werff, Henry. Antagonists of nucleic acid derivatives. I. The Lactobacillus casei model, 1

Hofmann, Klaus, Dickel, Daniel F., and Axelrod, A. E. The preliminary characterization of two biotin-containing fractions in beef liver,

Hogeboom, George H. See Schneider and Hogeboom,

Horecker, B. L. Triphosphopyridine nucleotide-cytochrome c reductase in

Huang, H. T. See Iselin, Huang, and 403 Niemann,

Iselin, Beat M., Huang, H. T., and Niemann, Carl. A colorimetric method for the determination of chymotrypsin 403 activity,

Jensen, H. See Cagan, Gray, and Jen-Johansson, R. See Baddiley, Ehrensvärd, Johansson, Reio, Saluste, and Stjernholm. Jorpes, J. Erik, Boström, Harry, and Mutt. Viktor. The linkage of the

amino group in heparin,

607

Karlsson, J. L. Metabolic studies of Azotobacter agilis by the use of a mutant deficient in pyruvic oxidase, 549 Kearney, Edna B. See Singer and

409 Kearney,

806 INDEX

Keating, Richard P. See Schulman and Keating, 21.
Keller, Elizabeth B., Boissonnas
R. A., and du Vigneaud, Vincent. The origin of the methyl group of epi
origin of the methyl group of epi
nephrine, 62°
See Simmonds, Keller, Chandler
and du Vianeaud. 19
Keresztesy, John C., and Silverman
Milton. Partial purification of
factor essential for growth of Leuco
nostoc citrovorum, 473
Kielley, W. Wayne, and Meyerhof, Otto
Studies on adenosinetriphosphatas
Studies on adenosinetriphosphatase of muscle. III. The lipoprotein
nature of the magnesium-activated
adanceinstriphoenhotees 30
adenosinetriphosphatase, 39. Kitchel, Mary F. See Harger, Raney
Reidwell and Kitchel
Bridwell, and Kitchel, 19 Klein, E. See Baddiley, Ehrensvärd
Klain Pain and Saluate 77
Klein, Reio, and Saluste, 77' Klendshoj, Niels C., Feldstein, Milton
and Sprague, Alice L. The spectro
photometric determination of carbon
monoxide, 29' Knoepfelmacher, Augusta A. See Vest
ling and Knoepfelmacher, 68 Kunkel, H. O., and Nelson, Walter L
The effect of bixin and carotene on the
oxidation of methyl linoleate, 14
Landua, Alton J. See Awapara, Landua
and Fuerst, 54
Levedahl, Blaine H. See Samuels
Sweat, Levedahl, Pottner, and Helm
reich, 23
Lifson, Nathan. See Lorber, Lifson
Sakami, and Wood, 53. — See Lorber, Lifson, Wood, Sakami
See Lorber, Lifson, Wood, Sakami
and Shreeve, 51'
Little, Henry N., and Bloch, Konrad
Studies on the utilization of acetic
acid for the biological synthesis of
cholesterol, 33
London, Irving M., Shemin, David
and Rittenberg, D. Synthesis of
heme in vitro by the immature non
nucleated mammalian arythropyte
heme in vitro by the immature non nucleated mammalian erythrocyte, 749
See Shemin, London, and Ritten
bera 757. 767

Lorber, Victor, Lifson, Nathan, Sakami, Warwick, and Wood, Harland G. Conversion of propionate to liver glycogen in the intact rat, studied with isotopic propionate, 531—, —, Wood, Harland G., Sakami, Warwick, and Shreeve, Walton W. Conversion of lactate to liver glycogen in the intact rat, studied with isotopic lactate, 517
Lubschez, Rose. Chemical determination of histamine in human blood, 731

Luebering, J. See Rapoport and Luebering, 507

M

Mackenzie, Cosmo G., Rachele, Julian R., Cross, Nancy, Chandler, Joseph P., and du Vigneaud, Vincent. A study of the rate of oxidation of the methyl group of dietary methionine,

Mackenzie, Julia B., Rosenkrantz, H.,
Ulick, Stanley, and Milhorat, Ade T.
The biological activity of α- tocopherylhydroquinone and α-tocopherylquinone,
655

Marlow, H. W. Groups involved in the Zimmermann and Kober reactions, 167

McConnell, Kenneth P., and Cooper, Betty J. Distribution of selenium in serum proteins and red blood cells after subcutaneous injection of sodium selenate containing radioselenium, 459

McGilvery, R. W., and Cohen, Philip P. Enzymatic synthesis of ornithuric acids, 179

McShan, W. H. See Copenhaver, Mc-Shan, and Meyer, 73

Meyer, Roland K. See Copenhaver, McShan, and Meyer, 73

Meyerhof, Otto, and Green, Harry.
Synthetic action of phosphatase. II.
Transphosphorylation by alkaline phosphatase in the absence of nucleotides,

377

See Kielley and Meyerhof, 391
 Milhorat, Ade T. See Mackenzie,
 Rosenkrantz, Ulick, and Milhorat, 655

AUTHORS 807

Mutt, Viktor. See Jorpes, Boström, and Mutt, 607	Pottner, M. M. See Samuels, Sweat, Levedahl, Pottner, and Helmreich,
N	231
Naganna, B. Erythrocyte pyrophos-	R Poblassita M and Power Poul P
phatase in health and disease. II. Reversible and irreversible inactiva-	Rabinovitz, M., and Boyer, Paul D. The inhibition of the succinoxidase
tions of the enzyme, 693	system by α -tocopheryl phosphate and
Nelson, Walter L. See Kunkel and	sodium dodecyl sulfate, 111
Nelson, 149	Rachele, Julian R. See Mackenzie,
Neimann, Carl. See Iselin, Huang, and	Rachele, Cross, Chandler, and du
Niemann, 403	Vigneaud, 617
	Raney, B. B. See Harger, Raney, Bridwell, and Kitchel, 197
Patterson, Helen R. See Engel, Patter-	Rapoport, S., and Luebering, J. The
son, Wilson, and Schinkel, 47	formation of 2,3-diphosphoglycerate
Patterson, John W. The diabetogenic effect of dehydroascorbic and dehy-	in rabbit erythrocytes: the existence of a diphosphoglycerate mutase,
droisoascorbic acids, 81	507
Person, P. See Eichel, Wainio, Person,	Rapport, David. See Canzanelli, Rap-
and Cooperstein, 89	port, and Guild, 291
Peterson, D. W. Some properties of a factor in alfalfa meal causing depres-	Rebstock, Mildred C. See Glazko, Dill,
sion of growth in chicks, 647	and Rebstock, 679 Reed, Lester J. The occurrence of
Philips, Frederick S. See Bendich,	γ-aminobutyric acid in yeast extract;
Brown, Philips, and Thiersch, 267	its isolation and identification,
Phillips, Robert A., Van Slyke, Donald	451
D., Hamilton, Paul B., Dole, Vincent	Reichard, Peter. See Hammarsten, Rei-
P., Emerson, Kendall, Jr., and Archi-	chard, and Saluste, 105
bald, Reginald M. Measurement of	Reio, L. See Baddiley, Ehrensvärd, Joh-
specific gravities of whole blood and plasma by standard copper sulfate	ansson, Reio, Saluste, and Stjernholm, 771
solutions, 305	See Baddiley, Ehrensvärd, Klein,
See Van Slyke, Hiller, Phillips,	Reio, and Saluste, 777
Hamilton, Dole, Archibald, and Eder,	Rittenberg, D. See London, Shemin,
331	and Rittenberg, 749
 See Van Slyke, Phillips, Dole, Hamilton, Archibald, and Plazin, 	—. See Shemin, London, and Rittenberg, 757, 767
349	Rodney, Gertrude, Swanson, Ann L.,
Pihl, Alexander, and Bloch, Konrad.	Wheeler, L. M., Smith, Grant N.,
The relative rates of metabolism of	and Worrel, Cecilia S. The effect of a
neutral fat and phospholipides in	series of flavonoids on hyaluronidase
various tissues of the rat, 431	and some other related enzymes, 739
-, -, and Anker, H. S. The rates of synthesis of fatty acids and cholesterol	Roll, Paul M. See Furst, Roll, and
in the adult rat studied with the aid of	Brown, 251 Rosenfeld, Morris, and Surgenor,
labeled acetic acid, 441	Douglas M. Methemalbumin. In-
Plazin, John. See Van Slyke, Phillips,	teraction between human serum albu-
Dole, Hamilton, Archibald, and	min and ferriprotoporphyrin IX, 663
Plazin, 349	Rosenkrantz, Harris. See Mackenzie,
Potter, Richard L., and Elvehjem, C. A.	Rosenkrantz, Ulick, and Milhorat,
On the activity of oxybiotin, 587	655

808 INDEX

Roth, Jay S., and Allison, James B.

The effects of feeding excess premethionine and choline chloride to rats on a casein diet,

173

Russell, Peter B. See Hitchings, Elion, Falco, Russell, Sherwood, and Vander-Werff, 1

Sakami, Warwick. See Lorber, Lifson, Sakami, and Wood, 531

See Lorber, Lifson, Wood, Sakami, and Shreeve,517

Saluste, E. See Baddiley, Ehrensvärd, Johansson, Reio, Saluste, and Stjernholm, 771

See Baddiley, Ehrensvärd, Klein,
 Reio, and Saluste,
 777

See Hammarsten, Reichard, and Saluste, 105

Samuels, Leo T., Sweat, Max L., Levedahl, Blaine H., Pottner, M. M., and Helmreich, M. L. Metabolism of testosterone by livers of different species of animals, 231

Schinkel, Marion. See Engel, Patterson, Wilson, and Schinkel, 47

Schneider, John J. Studies on the excretion of adrenocortical compounds. I. Isolation of 17-hydroxy-11-dehydrocorticosterone and other compounds from the urine of normal males, 365

Schneider, Walter C., and Hogeboom, George H. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat liver homogenates, 123

Schubert, Jack, and Wallace, Herschel, Jr. The effect of zirconium and sodium citrate on the distribution and excretion of simultaneously injected thorium and radiostrontium, 157

Schulman, John, Jr., and Keating, Richard P. Studies on the metabolism of thiourea. I. Distribution and excretion in the rat of thiourea labeled with radioactive sulfur, 215

Shantz, Edgar M., and Brinkman, John H. Biological activity of pure vitamin A₂, 467

Shemin, David, London, Irving M., and Rittenberg, D. The synthesis of protoporphyrin in vitro by red blood cells of the duck, 757

--, --, and --. The relationship of serine to porphyrin synthesis, 767
--. See London, Shemin, and Rittenberg,

749

Sherwood, Marion B. See Hitchings, Elion, Falco, Russell, Sherwood, and VanderWerff, 1

Shreeve, Walton W. See Lorber, Lifson, Wood, Sakami, and Shreeve, 517 Silverman, Milton, See Keresztesy and

Silverman, Milton. See Keresztesy and Silverman, 473

Simmonds, Sofia, Keller, Elizabeth B., Chandler, Joseph P., and du Vigneaud, Vincent. The effect of ethionine on transmethylation from methionine to choline and creatine in vivo, 191

Singer, Thomas P., and Kearney, Edna B. The non-enzymatic reduction of cytochrome c by pyridine nucleotides and its catalysis by various flavins,

Smith, Emil L., and Brown, Douglas M.
The sedimentation behavior of bovine and equine immune proteins, 241

Smith, Grant N. See Rodney, Swanson, Wheeler, Smith, and Worrel, 739

Spies, Joseph R., and Chambers, Dorris
 C. Utilization of optical isomers of methionine and formylmethionine by some lactobacilli,
 709

Sprague, Alice L. See Klendshoj, Feldstein, and Sprague, 297

Ställberg-Stenhagen, Stina, and Stenhagen, Einar. Studies on hydrocarbons structurally related to phthiocerol. Synthesis of the levorotatory enantiomorph of 4-methyltritriacontane, 223

Steinhardt, Jacinto, and Zaiser, Ethel M. Combination of wool protein with cations and hydroxyl ions, 789

Stenhagen, Einar. See Ställberg-Stenhagen and Stenhagen, 223

Stevens, Carl M., and Bush, Jess A. New syntheses of α-amino-ε-guanidinon-caproic acid (homoarginine) and its possible conversion in vivo into lysine,

139

AUTHORS 809

Stjernholm, R. See Baddiley, Ehrensvärd, Johansson, Reio, Saluste, and Stjernholm, 771
Surgenor, Douglas M. See Rosenfeld and Surgenor, 663
Swanson, Ann L. See Rodney, Swanson, Wheeler, Smith, and Worrel, 739
Sweat, Max L. See Samuels, Sweat,

Levedahl, Pottner, and Helmreich,
231

Thiersch, J. B. See Bendich, Brown, Philips, and Thiersch, 267

U

Ulick, Stanley. See Mackenzie, Rosenkrantz, Ulick, and Milhorat, 655

VanderWerff, Henry. See Hitchings, Elion, Falco, Russell, Sherwood, and VanderWerff,

Van Pilsum, John F., and Berg, Clarence P. The comparative availabilities of mixtures of the L and DL modifications of the essential amino acids for growth in the rat,

Van Slyke, Donald D., Hiller, Alma, Phillips, Robert A., Hamilton, Paul B., Dole, Vincent P., Archibald, Reginald M., and Eder, Howard A. The estimation of plasma protein concentration from plasma specific gravity, 331

Phillips, Robert A., Dole, Vincent P., Hamilton, Paul B., Archibald, Reginald M., and Plazin, John. Calculation of hemoglobin from blood specific gravities,

See Phillips, Van Slyke, Hamilton,
 Dole, Emerson, and Archibald, 305

Vestling, Carl S., and Knoepfelmacher,
Augusta A. Lactic dehydrogenase of
liver and its relation to thyroid
activity in the rat, 63
du Vigneaud, Vincent. See Keller,
Boissonnas, and du Vigneaud, 627
—. See Mackenzie, Rachele, Cross,
Chandler, and du Vigneaud, 617
—. See Simmonds, Keller, Chandler,
and du Vigneaud. 191

W

Wainio, W. W. See Eichel, Wainio, Person, and Cooperstein, 89 Wallace, Herschel, Jr. See Schubert and Wallace, 157 Wheeler, L. M. See Rodney, Swanson, Wheeler, Smith, and Worrel, 739

Williams, J. N., Jr., and Elvehjem, C. A. The effects of tryptophan deficiency upon enzyme activity in the rat, 539

Wilson, Hildegard. See Engel, Patterson, Wilson, and Schinkel, 47
Wood, Harland G. See Lorber, Lifson,

Sakami, and Wood, 531

—. See Lorber, Lifson, Wood, Sakami, and Shreeve, 517

Woodruff, H. Boyd, and Foster, John C. Analysis for vitamin B₁₂ and vitamin B_{12a} by paper strip chromatography, 569

Woolley, D. W. A study of the basis of selectivity of action of antimetabolites with analogues of pimelic acid, 495

Worrel, Cecilia S. See Rodney, Swanson, Wheeler, Smith, and Worrel,

Zaiser, Ethel M. See Steinhardt and Zaiser, 789

INDEX TO SUBJECTS

A	Alcohol(s)—continued:
Acetic acid: Cholesterol synthesis, bio-	Urine, neutral steroid, determination,
logical, Little and Bloch, 33	colorimetric, Engel, Patterson,
Indole See Indoleacetic acid	Wilson, and Schinkel, 47
Labeled, fatty acid and cholesterol	Water, determination and identifi-
synthesis, use in study, Pihl, Bloch,	cation, Harger, Raney, Bridwell, and
and Anker, 441	Kitchel, 197
Metabolism, Torulopsis utilis, Bad-	Alfalfa: Growth, chick, effect, Peterson,
diley, Ehrensvärd, Johansson, Reio,	. 647
Saluste, and Stjernholm, 771	Amino acid(s): DL-, growth, availabil-
Baddiley, Ehrensvärd, Klein, Reio,	ity, Van Pilsum and Berg, 279
and Saluste, 777	L-, growth, availability, Van Pilsum
Torulopsis utilis, tyrosine and, meta-	and Berg, 279
bolic relation, Baddiley, Ehrensvärd,	Oxidase, endocrine secretions, effect,
Klein, Reio, and Saluste, 777	Cagan, Gray, and Jensen, 11
Adenine: Oxidation in vivo, direct, Ben-	Aminobutyric acid: γ -, yeast, isolation
dich, Brown, Philips, and Thiersch,	and identification, Reed, 451
267	Aminoethylphosphoric ester: Free, tu-
Adenosinetriphosphatase: Muscle, Kiel-	mor and organs, Awapara, Landua,
ley and Meyerhof, 391	and Fuerst, 545
-, magnesium-activated, lipoprotein	Amino- ϵ -guanidino- n -caproic acid: α -,
nature, Kielley and Meyerhof, 391	conversion to lysine in vivo, Stevens
Adrenal(s): Cortex, compounds, ex-	and Bush, 139
cretion, Schneider, 365	-, synthesis, Stevens and Bush, 139
Adrenalin: See also Epinephrine	Arginine: Homo See Amino-e-guani-
Air: Alcohol, determination and identi-	dino-n-caproic acid
fication, Harger, Raney, Bridwell,	Ascorbic acid: Dehydro See Dehy-
and Kitchel, 197	droascorbic acid
Alanine: Synthesis, liver, in vitro, car-	Dehydroiso See Dehydroisoascorbic
	acid
bohydrate deprivation, effect, Can-	
zanelli, Rapport, and Guild, 291	Azotobacter agilis: Metabolism, pyruvic
Albumin: Blood serum, ferriprotopor-	oxidase-deficient mutant in study,
phyrin IX and, interaction, Rosen-	Karlsson, 549
feld and Surgenor, 663	В
Methem See Methemalbumin	В
Alcohol(s): Air, determination and	Bacillus: See also Lactobacillus
identification, Harger, Raney, Brid-	Bacteria: See also Azotobacter, Leuco-
well, and Kitchel, 197	nostoc
Blood, determination and identifi-	Biotin(s): Liver, characterization, Hof-
cation, Harger, Raney, Bridwell, and	mann, Dickel, and Axelrod, 481
Kitchel, 197	Oxy See Oxybiotin
Partition ratio, air and water, Harger,	Bixin: Methyl linoleate oxidation, ef-
Raney, Bridwell, and Kitchel, 197	fect, Kunkel and Nelson, 149
, urine and blood, Harger, Raney,	Blood: Alcohol, determination and
Bridwell, and Kitchel, 197	identification, Harger, Raney, Brid-
Urine, determination and identifica-	well, and Kitchel, 197
tion, Harger, Raney, Bridwell, and	Glucose, galactose administration, ef-
Kitchel, 197	fect, Greenman, 577

812 INDEX

Blood—continued:	
Histamine, determination, chemical,	
Lubschez, 731	
Specific gravity determination, copper	
sulfate solutions, use, Phillips, Van	
Slyke, Hamilton, Dole, Emerson, and	
Archibald, 305	
— —, hemoglobin determination, re-	
lation, Van Slyke, Phillips, Dole,	
Hamilton, Archibald, and Plazin,	
349	
Blood cell(s): Red, 2,3-diphospho-	
glycerate formation, Rapoport and	
Luebering, 507	
-, diphosphoglycerate mutase, Rapo-	
port and Luebering, 507	
-, immature non-nucleated, heme	
synthesis in vitro, London, Shemin,	
and Rittenberg, 749	
-, protoporphyrin synthesis in vitro,	
Shemin, London, and Rittenberg,	
757	
-, pyrophosphatase, Naganna, 693	
-, selenium, sodium radioselenate	
injection, effect, McConnell and	(
Cooper, 459	
Blood plasma: Protein, determination,	
blood plasma specific gravity, use,	
Van Slyke, Hiller, Phillips, Hamil-	
ton, Dole, Archibald, and Eder,	
331	
Specific gravity, blood plasma protein	
determination, use, Van Slyke,	(
Hiller, Phillips, Hamilton, Dole,	
Archibald, and Eder, 331	
, determination, copper sulfate	
solutions, use, Phillips, Van Slyke,	(
Hamilton, Dole, Emerson, and	
Archibald, 305	•
Blood serum: Albumin and ferriproto-	
porphyrin IX, interaction, Rosen-	
feld and Surgenor, 663	(
Protein, selenium, sodium radioselen-	
ate injection, effect, McConnell and	
Cooper, 459	(
Butyric acid: γ-Amino See Aminobu-	
tyric acid	
C	
Caproic acid: a - Amino - e - guanidino - n	(
See Amino $-\epsilon$ -guanidino $-n$ -caproic	

acid

Carbazole: Reaction, hexuronic acids. polyuronide study, Dische, Carbohydrate: Liver alanine synthesis, in vitro, effect, Canzanelli, Rapport, and Guild, Carbon monoxide: Determination, spectrophotometric. Klendshoj. Feldstein, and Sprague, 297 Carotene: Methyl linoleate oxidation. effect, Kunkel and Nelson, Casein: Choline chloride with, effect. Roth and Allison, 173 DL-Methionine with, effect, Roth and Allison, 173 Cation(s): Wool proteins and, combination, Steinhardt and Zaiser, 789 Chick: Growth, alfalfa, effect, Peterson, 647 Chloramphenicol: See Chloromycetin Chloromycetin: Biochemistry. Dill, and Rebstock, 679 Urine metabolic products, isolation and identification, Glazko, Dill, and Rebstock, 679 Chlorophyll: Synthesis, biological, magnesium vinyl pheoporphyrin as relation, Granick, Cholesterol: Synthesis, biological, acetic acid, utilization, Little and Bloch, - rate, labeled acetic acid in study,

- rate, labeled acetic acid in study, Pihl, Bloch, and Anker, 441 Choline: Methionine transmethylation

in vivo to, ethionine effect, Simmonds, Keller, Chandler, and du Vigneaud,

Choline chloride: Casein with, effect, Roth and Allison, 173

Chymotrypsin: Determination, colorimetric, Iselin, Huang, and Niemann, 403

Corticosterone: 17-Hydroxy-11-dehydro-. See Hydroxy-11-dehydrocorticosterone

Creatine: Methionine transmethylation in vivo to, ethionine effect, Simmonds, Keller, Chandler, and du Vigneaud, 191

Cystine: Urine, determination, Leuconostoc mesenteroides use, Camien and Dunn, 561

813

Cytochrome: b and cytochrome oxidase,	Enzyme(s)—continued:
separation and characterization,	Tryptophan deficiency, effect, Wil-
Eichel, Wainio, Person, and Cooper-	liams and Elvehjem, 539
stein, 89	See also Adenosinetriphosphatase,
c, catalysis by flavins, Singer and	Chymotrypsin, etc.
Kearney, 409	Epinephrine: Methyl group, origin,
-, liver, Schneider and Hogeboom, 123	Keller, Boissonnas, and du Vigneaud,
-, reductase, triphosphopyridine nu-	627
cleotide-, liver, Horecker, 593	Erythritol tetranitrate: Reduction, en-
-, reduction, non-enzymatic, pyri-	zymatic, glutathione effect, Heppel
dine nucleotide effect, Singer and	and Hilmoe, 129
Kearney, 409	Erythrocyte: See Blood cell, red
Oxidase and cytochrome b, separation	Ethionine: Methionine transmethyla-
and characterization, Eichel, Wai-	tion to choline and creatine in vivo,
nio, Person, and Cooperstein, 89	effect, Simmonds, Keller, Chandler,
nio, reroon, una cooperatein, co	and du Vigneaud, 191
D	tim at vigitation,
Dehydroascorbic acid: Diabetogenic ef-	
fect, Patterson, 81	Det(a) - Nontrol Dill
Dehydrogenase: Glutamic acid, tissue	Fat(s): Neutral, metabolism rate, Pihl
homogenates, determination, Copen-	and Bloch, 431
	Fatty acid(s): Synthesis rate, labeled
haver, McShan, and Meyer, 73	acetic acid in study, Pihl, Bloch,
Lactic, liver, thyroid activity, rela-	and Anker, 441
tion, Vestling and Knoepfelmacher,	Ferriprotoporphyrin: IX, blood serum
Bahadaslassaankis saida Diskatasanis	albumin and, interaction, Rosenfeld
Dehydroisoascorbic acid: Diabetogenic	and Surgenor, 663
effect, Patterson, 81	Flavin(s): Cytochrome c catalysis, ef-
Desoxypentose: Nucleic acid, purines,	fect, Singer and Kearney, 409
incorporation, Furst, Roll, and	Flavonoid(s): Hyaluronidase and re-
Brown, 251	lated enzymes, effect, Rodney,
Diabetes: Dehydroascorbic acid and	Swanson, Wheeler, Smith, and Wor-
dehydroisoascorbic acid, effect, Pat-	rel, 739
terson, 81	Formylmethionine: Optical isomers,
Diphosphoglycerate: 2,3-, formation,	utilization, lactobacilli, Spies and
blood cell, red, Rapoport and Lue-	Chambers, 709
bering, 507	G
Mutase, blood cell, red, Rapoport and	Catastana Diad alaman administra
Luebering, 507	Galactose: Blood glucose, administra-
E	tion effect, Greenman, 577
Endocrine(s): Secretions, amino acid	Glucose: Blood, galactose administra- tion. effect. Greenman. 577
oxidase, effect, Cagan, Gray, and	Glutamic acid: Dehydrogenase, tissue
Jensen, 11	homogenates, determination, Co-
Enzyme(s): Erythritol tetranitrate re-	penhaver, McShan, and Meyer, 73
duction, glutathione effect, Heppel	Glutathione: Nitroglycerin and eryth-
and Hilmoe, 129	ritol tetranitrate reduction, enzy-
Intracellular distribution, Schneider	matic, effect, Heppel and Hilmoe,
and Hogeboom, 123	129
Nitroglycerin reduction, glutathione	Glycogen: Liver, lactate conversion to,
effect, Heppel and Hilmoe, 129	isotopic lactate in study, Lorber,
Ornithuric acid synthesis, McGilvery	Lifson, Wood, Sakami, and Shreeve,
and Cohen, 179	517

Glycogen—continued:	K
Liver, propionate conversion to, iso-	Kober reaction: Groups involved
topic propionate in study, Lorber,	Marlow, 167
Lifson, Sakami, and Wood, 531	mariow,
Growth: Alfalfa, chick, effect, Peterson,	L
647	Lactate: Liver glycogen formation from
DL-Amino acids, availability, Van Pil-	isotopic lactate in study, Lorber
sum and Berg, 279	
L-Amino acids, availability, Van Pil-	Lifson, Wood, Sakami, and Shreeve
sum and Berg, 279	517
• /	Lactic dehydrogenase: Liver, thyroid
H	activity, relation, Vestling and
Heme: Synthesis, blood cell, red, im-	Knoepfelmacher, 63
mature non-nucleated, in vitro,	Lactobacillus: Methionine and formyl-
	methionine optical isomers, utiliza-
London, Shemin, and Rittenberg,	tion, Spies and Chambers, 709
749	Lactobacillus casei: Nucleic acid de-
Hemoglobin: Determination, blood spe-	rivatives, effect, Hitchings, Elion,
cific gravity relation, Van Slyke,	Falco, Russell, Sherwood, and Van-
Phillips, Dole, Hamilton, Archibald,	derWer,ff,
and Plazin, 349	Leuconostoc citrovorum: Growth fac-
Heparin: Amino group linkage, Jorpes,	tor, purification, Keresztesy and Sil-
Boström, and Mutt, 607	verman, 473
Hexuronic acid(s): Carbazole reaction,	Leuconostoc mesenteroides: Urine cys-
polyuronide study, Dische, 489	tine determination, use, Camien and
Histamine: Blood, determination,	Dunn, 561
chemical, Lubschez, 731	Lipide(s): Phospho See Phospholip-
Homoarginine: See Amino-ε-guanidino-	ide
n-caproic acid	Lipoprotein: Muscle, magnesium-acti-
Hormone(s): Plant, substituted indole-	vated adenosinetriphosphatase, re-
acetic acids as, pea test, Findlay	lation, Kielley and Meyerhof, 391
and Dougherty, 361	Liver: Alanine synthesis in vitro, car-
Hyaluronidase: Flavonoids, effect,	bohydrate deprivation, effect, Can-
Rodney, Swanson, Wheeler, Smith,	zanelli, Rapport, and Guild, 291
and Worrel, 739	Biotins, characterization, Hofmann,
-Related enzymes, flavonoids, effect,	Dickel, and Axelrod, 481
Rodney, Swanson, Wheeler, Smith,	Cytochrome c, Schneider and Hoge-
and Worrel, 739	boom, 123
Hydrocarbon (s): Phthiocerol - related,	Glycogen, lactate conversion to, iso-
Ställberg-Stenhagen and Stenhagen,	topic lactate in study, Lorber, Lif-
223	son, Wood, Sakami, and Shreeve,
Hydroxy-11-dehydrocorticosterone: 17-,	517
urine, isolation, Schneider, 365	, propionate conversion to, isotopic
Hydroxyl: Ions, wool protein and, com-	propionate in study, Lorber, Lifson,
bination, Steinhardt and Zaiser,	Sakami, and Wood, 531
789	Lactic dehydrogenase, thyroid activ-
_	ity, relation, Vestling and Knoep-
I	felmacher, 63
Indoleacetic acid(s): Substituted as	Testosterone metabolism, Samuels,
plant hormones, pea test, Findlay	Sweat, Levedahl, Pottner, and Helm-
and Dougherty, 361	reich, 231

SUBJECTS 815

Times and to said	Wasters Dil G Dil
Liver—continued:	Nuclease: Ribo See Ribonuclease
Triphosphopyridine nucleotide-cyto-	Nucleic acid: Derivatives, antagonists,
chrome c reductase, Horecker,	Hitchings, Elion, Falco, Russell,
593	Sherwood, and VanderWerff, 1
Lysine: α-Amino-e-guanidino-n-caproic	-, Lactobacillus casei effect, Hitchings,
acid conversion to, in vivo, Stevens	Elion, Falco, Russell, Sherwood,
and Bush, 139	and VanderWerff, 1
M	Desoxypentose, purines, incorpora-
Magnesium: -Activated adenosinetri-	tion, Furst, Roll, and Brown,
phosphatase, muscle, lipoprotein	Pantaga mananan asast sibasashia
nature, Kielley and Meyerhof, 391	Pentose, pancreas, yeast ribonucleic
Vinyl pheoporphyrin as, chlorophyll	acid and, comparison, Bacher and
biosynthesis, relation, Granick,	Allen, 641
713	, purines, incorporation, Furst,
Methemalbumin: Rosenfeld and Surge-	Roll, and Brown, 251
nor, 663	Ribo. See Ribonucleic acid
Methionine: DL-, casein with, effect,	Nucleoside(s): Pyrimidine, polynucleo-
Roth and Allison, 173	tide pyrimidines, precursors, Ham-
Formyl See Formylmethionine	marsten, Reichard, and Saluste,
Methyl group, oxidation, Mackenzie,	105
Rachele, Cross, Chandler, and du	Nucleotide(s): Poly See Polynucle-
Vigneaud, 617	otide
Optical isomers, utilization, lactoba-	Pyridine, cytochrome c reduction,
cilli, Spies and Chambers, 709	non-enzymatic, effect, Singer and
Transmethylation to choline and cre-	Kearney, 409
atine in vivo, ethionine effect, Sim-	Triphosphopyridine, -cytochrome c
monds, Keller, Chandler, and du	reductase, liver, Horecker, 593
Vigneaud, 191	
Methyl linoleate: Oxidation, bixin and	О
carotene effect, Kunkel and Nelson,	Ornithuric acid: Synthesis, enzymatic,
149	McGilvery and Cohen, 179
Methyl tritriacontane: 4-, synthesis,	Oxidase: Amino acid, endocrine secre-
Ställberg-Stenhagen and Stenhagen,	tions, effect, Cagan, Gray, and Jen-
223	sen, 11
Muscle: Adenosinetriphosphatase, Kiel-	Cytochrome oxidase and cytochrome
ley and Meyerhof, 391	b, separation and characterization,
-, magnesium-activated, lipoprotein	Eichel, Wainio, Person, and Cooper-
nature, Kielley and Meyerhof, 391	stein, 89
Mutase: Diphosphoglycerate, blood	Pyruvic, mutant deficient in, Azoto-
cell, red, Rapoport and Luebering,	bacter agilis metabolism, use in
507	study, Karlsson, 549
N SS.	Succin See Succinoxidase
- ·	Oxybiotin: Activity, Potter and Eleve-
Nitrate ester(s): Inorganic, metabo-	hjem, 587
lism, Heppel and Hilmoe, 129	,
Nitrite ester(s): Inorganic, metabolism,	~

129

Heppel and Hilmoe,

Hilmoe,

Nitroglycerin: Reduction, enzymatic,

glutathione effect, Heppel

P

Pancreas: Pentose nucleic acid, yeast ribonucleic acid and, comparison, Bacher and Allen,

Pea: Test, indoleacetic acids, substi-

Protein(s): Blood plasma, determina-

Loss, Mudicacent actus, Bubbit	Trotter (a) . Diood branna, deferriting-
tuted, as plant hormones, Findlay	tion, blood plasma specific gravity,
and Dougherty, 361	use, Van Slyke, Hiller, Phillips,
Pentose: Desoxy See Desoxypentose	Hamilton, Dole, Archibald, and Eder,
Nucleic acid, pancreas, yeast ribonu-	331
cleic acid and, comparison, Bacher	, — serum, selenium, sodium radiosel-
and Allen, 641	enate injection, effect, McConnell
-, purines, incorporation, Furst,	and Cooper, 459
Roll, and Brown, 251	Immune, sedimentation studies, Smith
Pheoporphyrin: a _s , magnesium vinyl,	and Brown, 241
chlorophyll biosynthesis, relation,	Wool, cations and, combination,
Granick, 713	Steinhardt and Zaiser, 789
Phosphatase: Adenosinetri See	-, hydroxyl ions and, combination,
Adenosinetriphosphatase	Steinhardt and Zaiser, 789
Alkaline, transphosphorylation, nucle-	Protoporphyrin: Synthesis, blood cells,
otide absence, Meyerhof and Green,	red, in vitro, Shemin, London, and
377	Rittenberg, 757
Pyro See Pyrophosphatase	Purine(s): Desoxypentose nucleic acid,
Synthesis, Meyerhof and Green, 377	incorporation, Furst, Roll, and
Phosphoglycerate: Di See Diphos-	Brown, 251
phoglycerate	Pentose nucleic acid, incorporation,
Phospholipide(s): Metabolism rate,	Furst, Roll, and Brown, 251
Pihl and Bloch, 431	Pyridine: Nucleotide, cytochrome c re-
Phosphoric ester: Aminoethyl Sec	duction, non-enzymatic, effect,
Aminoethylphosphoric ester	Singer and Kearney, 409
Phthiocerol: Hydrocarbons structur-	Pyrimidine: Nucleosides, polynucleo-
ally related to, Ställberg-Stenhagen	tide pyrimidines, precursors, Ham-
and Stenhagen, 223	marsten, Reichard, and Saluste,
Pimelic acid: Analogues, antimetabo-	105
lites and, Woolley, 495	Pyrophosphatase: Blood cell, red, Naganna. 693
Plant: Hormones, substituted indole-	<i>3</i> ,
acetic acids as, pea test, Findlay and Dougherty. 361	Pyruvic oxidase: -Deficient mutant,
and Dougherty, 361 Polynucleotide(s): Pyrimidine, pyrimi-	Azotobacter agilis metabolism, use in study. Karlsson. 549
dine nucleosides as precursors,	study, Karlsson, 549
Hammarsten, Reichard, and Saluste,	0
105	•
	Quinone: \(\alpha\)-Tocopheryl See Tocoph-
Polyuronide (s): Hexuronic acid carbazole reaction, use in study, <i>Dische</i> ,	erylquinone
zoie reaction, use in study, Dische,	α-Tocopherylhydro See Tocopher-
Descharing Forming to See Forming to	ylhydroquinone
Porphyrin: Ferriproto See Ferriproto-	R
porphyrin Pheo See Pheoporphyrin	
Proto See Protoporphyrin	Reductase: Cytochrome c, triphospho-
	pyridine nucleotide-, liver, Horecker,
Synthesis, serine relation, Shemin,	593
London, and Rittenberg, 767	Ribonuclease: Yeast ribonucleic acid,
Propionate: Liver glycogen formation	effect, Bacher and Allen, 633
from, isotopic propionate in study,	Ribonucleic acid: Yeast, pancreas
Lorber, Lifson, Sakami, and Wood,	pentose nucleic acid and, compar-
531	ison, Bacher and Allen, 641

817

Ribonucleic acid—continued:	T
Yeast, ribonuclease effect, Bacher and	Testosterone: Metabolism, liver, Samu-
Allen, 633	els, Sweat, Levedahl, Pottner, and
S	Helmreich, 231
•	Thiourea: Metabolism, Schulman and
Selenium: Blood cell, red, sodium ra-	Keating, 215
dioselenate injection, effect, McCon-	Radioactive sulfur-labeled, distribu-
nell and Cooper, 459	tion and excretion, Schulman and Keating. 215
. — serum protein, sodium radioselen- ate injection, effect, McConnell and	Keating, 215 Thorium: Sodium citrate effect, Schu-
120	bert and Wallace, 157
Cooper, 459 Serine: Porphyrin symphesis, relation,	Zirconium effect, Schubert and Wallace,
Shemin, London, and Rittenberg,	157
767	Thyroid: Liver, lactic dehydrogenase,
Sodium citrate: Thorium and radio-	relation, Vestling and Knoepfel-
strontium injection, effect, Schubert	macher, 63
and Wallace, 157	Tocopherylhydroquinone: α -, biological
Sodium dodecyl sulfate: Succinoxidase	activity, Mackenzie, Rosenkrantz,
system, effect, Rabinovitz and Boyer,	Ulick, and Milhorat, 655
111 	Tocopheryl phosphate: α-, succinoxidase
Sodium selenate: Radioselenium-con-	system, effect, Rabinovitz and Boyer,
taining, blood serum protein and red blood cell selenium, injection effect,	Tocopherylquinone: α -, biological activ-
McConnell and Cooper, 459	ity, Mackenzie, Rosenkrantz, Ulick,
Specific gravity: Blood and blood	and Milhorat, 655
plasma, determination, copper sul-	Torulopsis utilis: Acetic acid and tyro-
fate solutions, use, Phillips, Van	sine, metabolic relation, Baddiley,
Slyke, Hamilton, Dole, Emerson,	Ehrensvärd, Klein, Reio, and Saluste,
and Archibald, 305	777
, hemoglobin determination, rela-	- metabolism, Baddiley, Ehrens-
tion, Van Slyke, Phillips, Dole, Ham-	vard, Johansson, Reio, Saluste, and
ilton, Archibald, and Plazin, 349	Stjernholm, 771
 plasma protein, blood plasma de- termination, use, Van Slyke, Hiller, 	Baddiley, Ehrensvärd, Klein, Reio, and Saluste, 777
Phillips, Hamilton, Dole, Archibald,	Cultivation, C12H2C14OOH as carbon
and Eder, 331	source, Baddiley, Ehrensvärd, Johans-
Steroid(s): Urine, neutral, alcohols, de-	son, Reio, Saluste, and Stjernholm,
termination, colorimetric, Engel,	771
Patterson, Wilson, and Schinkel,	Transphosphorylation: Phosphatase, al-
47	kaline, nucleotide absence, Meyerhof
Strontium: Radioactive, zirconium and	and Green, 377
sodium citrate effect, Schubert and	Triphosphopyridine: Nucleotide-cyto-
Wallace, 157	chrome c reductase, liver, Horecker,
Succinoxidase: System, \(\alpha\)-tocopheryl	593 Trypsin: Chymo See Chymotrypsin
phosphate and sodium dodecyl sul-	Tryptophan: Deficiency, enzymes, ef-
fate effect, Rabinovitz and Boyer, 111	fect, Williams and Elvehjem, 589
Sulfur: Radioactive, in thiourea, distri-	Tumor: Aminoethylphosphoric ester,
bution and excretion, Schulman and	free, Awapara, Landua, and Fuerst,
Kealing, 215	545

Tyrosine: Phenolic ring struct	ure, deg-
radation, Baddiley, El	
Klein, Reio, and Saluste,	777
Torulopsis utilis, acetic a	eid and,
metabolic relation, Baddiley	
värd, Klein, Reio, and Salust	e, 777

U

Urea: Thio -. See Thiourea Uric acid: Fate, Geren, Bendich, Bodansky, and Brown, Urine: Alcohol, determination and identification, Harger, Raney, Bridwell, and Kitchel. Alcohols, steroid, neutral, determination, colorimetric, Engel, Patterson, Wilson, and Schinkel, Chloromycetin metabolic products, isolation and identification, Glazko, Dill, and Rebstock, Cystine, determination, Leuconostoc mesenteroides use, Camien and Dunn, 561 17-Hydroxy-11-dehydrocorticosterone, isolation, Schneider, 365

Vitamin(s): A₂, biological activity, Shantz and Brinkman, 467 Vitamin(s)—continued:

B₁₂, determination, chromatographic,
Woodruff and Foster,

B_{12n}, determination, chromatographic,
Woodruff and Foster,

569

W

Water: Alcohol, determination and identification, Harger, Raney, Bridwell, and Kitchel, 197

Wool: Protein, cations and hydroxyl ions, combination, Steinhardt and Zaiser, 789

Y

Yeast: γ-Aminobutyric acid, isolation and identification, Reed, 451
Ribonucleic acid, pancreas pentose nucleic acid and, comparison, Bacher and Allen, 641
—, ribonuclease effect, Bacher and Allen, 633

See also Torulopsis

Zimmermann reaction: Groups involved, Marlow, 167
Zirconium: Thorium and radiostrontium injection, effect, Schubert and Wallace, 157